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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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MARINE BIOLOGICAL

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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Forty-Second Year

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MARINE BIOLOGICAL WOODS LIBRARY

II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[seal]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio,* who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeriti, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeriti shall each have the same right to vote as the regular Trustees.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen: Herewith is submitted my report as Treasurer of the Marine Biological Laboratory.

The accounts have been audited by Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

The book value of the General Endowment Fund in the hands of the Central Hanover Bank and Trust Company (of New York) as Trustees, was $908,145 in securities and $804.50 in cash.

A new trust known as the Library Endowment Fund was created by the gift on December 14th, of $200,000 from the General Education Board, and is also in the hands of the Central Hanover Bank and Trust Company. At the end of the year it was invested in bonds to the extent of $80,865 and the cash balance was $119,135. This was shortly thereafter invested to yield about five per cent (5%).

At the end of the year the Lucretia Crocker Fund consisted of securities of the book value of $4,442.46 and cash of $537.20.

The Ida H. Hyde Scholarship Fund, which consisted of $2,000 in a mortgage participation and interest, has been, with the consent of all parties, returned to Dr. Hyde, who has turned the Fund over to the University of Kansas for the benefit of whose students the Fund was established.

The Bio Club Scholarship Fund consisted of a $2,000 participation in a mortgage and cash of $23.28, and the Reynold A. Spaeth Memorial Lecture Fund consisted of a $3,000 participation in a mortgage and cash of $1.83.

The Retirement Fund at the end of the year consisted of $13,000 in mortgage participations and cash of $14.27. The income is approximately $650, the annual addition (5% of the permanent annual payroll) amounts to about $2,600 and the pension payments now authorized are at the rate of $720 annually.

The land, building, equipment and library, excluding the Devil's Lane and Gansett property, represented an investment of $1,600,352.93—less depreciation of $208,722.91—or a net amount of $1,391,630.02.

Current income for the year was exceeded by expenditures including depreciation by $855.33.

Over $20,000 was expended from current funds on buildings, equipment, books and reduction of mortgages.

At the end of the year the Laboratory owed less than $1,500 in accounts payable, and $27,000 on mortgages.

Following is the Balance Sheet at the end of the year, and the condensed statement of income and outgo for the year, also the surplus account.

EXHIBIT A

Marine Biological Laboratory Balance Sheet,

December 31, 1929

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank & Trust Company (of New York) Trustee—Schedules I-a and I-b		$1,108,949.50	
Securities and Cash—Minor Funds—Schedule II		12,049.77	$1,120,999.27
Plant Assets:			
Land—Schedule IV	$ 115,553.05		
Buildings—Schedule IV	1,206,242.06		
Equipment—Schedule IV	149,958.05		
Library—Schedule IV	128,599.77	$1,600,352.93	
Less Reserve for Depreciation		208,722.91	
		$1,391,630.02	
Cash in Dormitory Building Funds		1,451.76	$1,393,081.78
Current Assets:			
Cash		$ 17,144.45	
Accounts—Receivable		17,648.13	
Inventories:			
Supply Department	$ 35,850.39		
Biological Bulletin	7,461.00	43,311.39	
Investments:			
Devil's Lane Property	$ 36,260.01		
Gansett Property	2,076.30		
Stock in General Biological Supply House, Inc.	12,700.00		
Retirement Fund Assets	13,014.27	64,050.58	
Prepaid Insurance		3,986.80	
Items in Suspense		256.42	146,397.77

Liabilities

Endowment Funds:		
General Endowment Funds—Schedule III	$1,108,949.50	
Minor Endowment Funds—Schedule III	12,049.77	$1,120,999.27
Plant Funds:		
Donations and Gifts—Schedule III	$1,019,840.41	
Other Investments in Plant from Gifts and Current Funds	371,241.37	
	$1,391,081.78	
Mortgage, Danchakoff Estate	2,000.00	$1,393,081.78

Current Liabilities and Surplus:		
Mortgage, Devil's Lane Property	$ 25,000.00	
Accounts—Payable	1,464.48	
	$ 26,464.48	
Current Surplus—Exhibit C	119.933.29	146,397.77

EXHIBIT B

Marine Biological Laboratory Income and Expense, Year Ended December 31, 1929

	Total		Net	
	Expense	Income	Expense	Income
Income, General Endowment Fund		$ 46,549.09		$ 46,549.09
Income, Fund for Library, etc.	793.95	4,582.20		3,788.25
Gifts (See Current Surplus)				
Instruction	7,560.82	9,400.00		1,839.18
Research	4,073.91	16,625.00		12,551.09
Biological Bulletin and Membership Dues	6,288.58	8,025.93		1,737.35
Supply Department—Schedule V	52,611.28	62,056.83		9,445.55
Mess, Schedule VI	33,038.05	34,581.67		1,543.62
Dormitories, Schedule VII	31,386.60	13,754.81	17,631.79	
(Interest and Depreciation charged to above three Depts. See Schedules V, VI, and VII)	35,366.31			35,366.31
Dividends on Stock, General Biological Supply House, Inc.		2,540.00		2,540.00
Rent, Danchakoff Cottages	430.53	900.00		469.47
Rent, Microscopes		335.00		335.00
Rent, Garage, Railway, etc.		163.90		163.90
Rent, Newman Cottage	157.03	150.00	7.03	
Sale of Duplicate Library Sets		475.00		475.00
Interest on Bank Balances		267.63		267.63
Sundry Items		1.85		1.85
Maintenance of Plant:				
New Laboratory Expense	15,234.18		15,234.18	
Maintenance, Buildings and Grounds	10,399.72		10,399.72	
Chemical and Special Apparatus	9,144.10		9,144.10	
Library Department Expenses	8,894.07		8,894.07	
Carpenter Department Expenses	1,587.80		1,587.80	
Truck Expenses	800.47		800.47	
Sundry Expenses	148.18		148.18	
Bar Neck Property Expenses	381.00		381.00	
Evening Lectures	120.09		120.09	

Workmen's Compensation Insurance	598.23		598.23	
Janitor's House	289.42		289.42	
Pumping Station	267.49		267.49	
General Expenses:				
Administration Expenses	13,649.48		13,649.48	
Interest on Loans	259.00		259.00	
Endowment Trust Fund	787.50		787.50	
Bad Debts	523.96		523.96	
Contribution for Research, Naples Zoological Station	250.00		250.00	
Reserve for Depreciation	36,955.11		36,955.11	
Excess of Expense over Income carried to Current Surplus—Exhibit C		855.33		855.33
	$201,264.24	$201,264.24	$117,928.62	$117,928.62

EXHIBIT C

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT

YEAR ENDED DECEMBER 31, 1929

Balance, January 1, 1929		$100,637.23
Add:		
Gifts Received		
From General Education Board for Purchase of Books for Library		10,000.00
From Dr. Frank R. Lillie for Grading, Planting Walks, etc., around Laboratory Buildings		1,950.00
Income from Retirement Fund		305.04
Reserve for Depreciation charged to Plant Funds		36,955.11
		$149,847.38
Deduct:		
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,		
Buildings	$ 1,094.23	
Equipment	5,638.67	
Library Books, etc.	9,218.54	
	$15,951.44	
Payments from above Gifts charged to Plant Assets,		
General Education Board, Purchase of Books	6,657.32	
Dr. Frank R Lillie, Grading, etc.	1,950.00	
Payments on Mortgages,		
Danchakoff Estate	4,500.00	
Balance of Income-and-Expense Account—Exhibit B	855.33	29,914.09
Balance, December 31, 1929—Exhibit A		$119,933.29

Respectfully submitted,

LAWRASON RIGGS, JR.,

Treasurer.

V. THE REPORT OF THE LIBRARIAN

The close of the year 1929 marks a new period for the Library. The $50,000 presented by the General Education Board has become an integral part of the Library in the form of serial and monograph sets. A Library Endowment Fund of $200,000 by the same donor has been invested by the Treasurer of the Laboratory, the interest to form part of the annual Library budget. Besides this, and for the year 1929, a sum of $4,582.20, interest accumulated between the gift date and the actual transfer to the Laboratory, is now practically covered by outstanding orders.

With a steady income assured by this endowment fund, it becomes possible to plan a yearly budget, proportioned approximately as follows:

Books	$ 300
Serials	5,000
Binding	1,400
Back Sets (Serials and Reprints)	8,200
Express	300
Supplies	500
Salaries	8,300
Total	$24,000

The most notable feature brought out in contrast to past years is that current serials heretofore confined strictly to the Laboratory budget and not benefited by the special gift of the General Education Board will now share largely in the interest from the permanent endowment, and will form a total item of expenditure just about equal each year to the sum spent on back sets.

For convenience of future reference, etc., we would like to print here our full report of expenditures of the $50,000 gift just as these were reported each of the four years to the General Education Board. But the score of pages entailed scarcely justifies this procedure and the list may be consulted at any time in the Library files.

Besides that part of the above-mentioned figures for 1929, the expenditures: Books, $300.43; Serials, $4,787.19; Binding, $1,232.36; Express, $253.23; Supplies, $558.34; Salaries, $8124.18; total, $15,253.73, show an overrun of the budget ($14,000) amounting to $1,253.73, in accordance with the expected (see Report of the Library, 1927 and 1928). A sum of $929 from the sale of accumulated duplicates, etc., almost covers this and eventually (early in 1930), the Library will cancel the remaining deficit by the same means.

The Library starts on its new period with 23,455 bound serials; 4,855 books; and 58,658 reprints. A comparison with the 1928 report will give the 1929 actual figures of accretion. The serials now show

NE BIOLOGICAL LABORA
WOODS HOLE
LIBRARY

1,537 titles; of these 985 are received currently: 322 by subscription; 420 by exchange; and 253 by gift.

The Librarian wishes to acknowledge in the name of the Marine Biological Laboratory the following gifts to the Library:

From the authors: Addison, W. H. F. (Editor): *Piersol's Normal Histology;* Conn, H. J.: *Biological Stains;* Correns, Carl: *Gesammelte Abhandlungen zur Vererbungswissenschaft aus Periodischen Schriften, 1899–1924;* Goldschmidt, Richard: *Der Mendelismus, in Elementarer Darstellung;* Hance, Robert T.: *The Machines We Are;* Heilbrunn, L. V.: *The Colloid Chemistry of Protoplasm;* Lumière, Auguste: *Le Cancer, Maladie des Cicatrices;* Lumière, Auguste: *Le Problème de l'Anaphylaxie;* Lumière, Auguste: *La Vie, la Maladie et la Mort;* Patten, Bradley M.: *The Early Embryology of the Chick;* Schafer, Edward Sharpey: *History of the Physiological Society during its First Fifty Years, 1876–1926;* Whiting, P. W.: *A Series of Eight Radio Talks on Heredity and Human Problems;* Williams, Samuel Howard: *The Mammals of Pennsylvania;* Woodruff, Lorande L.: *Foundations of Biology.*

From the publishers:

Baillière, Tindall & Cox; Gaskell, Augusta: *What is Life?*

Thos. Y. Crowell Co.; Papez, James W.: *Comparative Neurology;* Wheeler, Raymond H.: *The Science of Psychology.*

Ginn and Co.; Edison, Oskar E. and Norris, F. W.: *Electrical Engineering Laboratory Practice;* Pierce, B. O.: *Short Tables of Integrals.*

Harvard University Press; Banks, Nathan and Myers, J. G.: *Studies on Cuban Insects, I.*

Paul B. Hoeber; Gross, Louis: *The Blood Supply to the Heart;* McClung, C. E. (Editor): *Handbook of Microscopical Technique;* Roth, Irving R.: *Cardiac Arrhythmias;* Stone, Willard J.: *Blood Chemistry, Colorimetric Methods;* Wright, Wilhelmine G.: *Muscle Function.*

Johns Hopkins Press; Camerson, Jenks: *The Bureau of Biological Survey;* Powell, Fred W.: *The Bureau of Plant Industry.*

Alfred A. Knopf, Inc.; Knickerbocker, Wm. S. (Editor): *Classics of Modern Science;* Nordenskiöld, Erik: *The History of Biology;* Wheeler, Wm. M.: *Foibles of Insects and Men.*

Lea & Febiger; Faust, Ernest Caroll: *Human Helminthology;* Kuntz, Albert: *The Autonomic Nervous System.*

McGraw-Hill Book Co.; Babcock, Ernest B. and Collins, J. L.: *Genetics, Laboratory Manual;* Daniels, F.: *Mathematical Preparation for Physical Chemistry;* Gäumann, Ernst A.: *Comparative Morphology of Fungi;* Goodwin, H. M.: *Precision of Measurements and Graphical Methods;* Joffe, Abram F.: *Physics of Crystals;* Knowlton, A. A.:

Physics for College Students; Metcalf, C. L. and Flint, W. P.: *Destructive and Useful Insects; Their Habits and Control;* Richtmyer, F. K.: *Introduction to Modern Physics;* Weaver, John E. and Bruner, Wm. E.: *Root Development of Vegetable Crops;* Weiser, Harry B.: *The Colloidal Salts.*

Macmillan Co.; Carpenter, Kathleen E.: *Life in Inland Waters with Especial Reference to Animals;* Coghill, G. E.: *Anatomy and the Problem of Behaviour;* Darwin, Charles: *The Origin of Species;* deBeer, G. R.: *Vertebrate Zoology;* Holmes, Samuel J.: *The Biology of the Frog;* Lindsey, Arthur Ward: *Textbook of Evolution and Genetics;* Walter, H. E.: *Biology of the Vertebrates;* Walter, H. E.: *Genetics;* Washburn, M. F.: *Animal Mind.*

Wm. Morrow & Co.; Stanford, Alfred: *Invitation to Danger.*

Museo Nacional de Ciencias National, Madrid: Lozano, Luis Rey: *Peces (Fauna Ibérica), vol. 1.*

G. P. Putnam's Son, Ltd.; Anthony, H. E.: *The Field Book of North American Mammals;* Breder, Charles M., Jr.: *Field Book of Marine Fishes of the Atlantic Coast.*

W. B. Saunders Co.; Dorland, W. A. Newman: *The American Illustrated Medical Dictionary;* Granger, Frank Butler: *Physical Therapeutic Technic;* Jordan, Edwin O.: *A Text-book of General Bacteriology;* Lusk, Graham: *The Elements of the Science of Nutrition;* Stiles, Percy G.: *Human Physiology;* Willius, Frederick A.: *Clinical Electrocardiograms, Their Interpretation and Significance.*

Smith College; Funkhouser, W. D.: *General Catalogue of the Hemiptera, Fasc. I.*

Charles C. Thomas, Publisher; Ewing, Henry Ellsworth: *A Manual of External Parasites;* Friedmann, Herbert: *The Cowbirds;* Needham, James G.: *Elementary Lessons on Insects;* Needham, James G. and Heywood, H. B.: *A Handbook of the Dragonflies of North America.*

University of California Press: Daniel, J. Frank: *The Elasmobranch Fishes;* Grinnell, Joseph & Storer, T. I.: *Animal Life in the Yosemite;* Grinnell, Joseph; Brayant, H. C., and Storer, T. I.: *The Game Birds of California;* Russell, E. J.: *Plant Nutrition and Crop Production.*

University of Chicago Press; Choulant, Ludwig: *History and Bibliography of Anatomy Illustration;* Michelson, A. A.: *Studies in Optics;* Morse, Jared Kirtland: *Bibliography of Crystal Structure;* Ornstein, Martha; *The Rôle of Scientific Societies in the Seventeenth Century.*

University of Colorado Press; Cockerell, T. D. A.: *Zoölogy of Colorado;* George, R. D.: *Geology and Natural Resources of Colorado;* Ramaley, F.: *Colorado Plant Life.*

D. Van Nostrand Co.; Robbins, Wm. J. and Rickett, Harold W.:

Botany, a Textbook for College and University Students; Robbins, W. J. and Rickett, Harold W.: *Laboratory Instructions for General Botany.*

Victor X-Ray Corporation; Jerman: *Modern X-Ray Technic.*

John Wiley & Sons; Pearse, A. S. and Hall, F. G.: *Homoiothermism, the Origin of Warm-Blooded Vertebrates;* Shumway, Waldo: *Vertebrate Embryology.*

Williams and Wilkins Co.; Bailey, Vernon: *Animal Life of the Carlsbad Cavern;* Brasch, Frederick E.: *Sir Isaac Newton, 1727–1927;* Brouwer, B.: *Anatomical, Phylogenetical and Clinical Studies on the Central Nervous System.*

Wisconsin Academy of Science; Baker, Frank C.: *Fresh Water Mollusca of Wisconsin.*

William Wood & Co.; Bailey, R. B. and Miller, A. M.: *Textbook of Embryology;* Stedman: *Medical Dictionary.*

Yale University Press; Brown, William: *Science and Personality;* Chittenden, Russell H.: *History of the Sheffield Science School of Yale University, 1846–1922;* Crawford, Albert Beecher: *Incentives to Study;* Eddington, A. S.: *Stars and Atoms;* Henderson, L. J.: *Blood, A Study in General Physiology;* Henderson, Yandell and Davie, M. R.: *Incomes and Living Costs of a University Faculty;* Krogh, August: *Anatomy and Phsyiology of Capillaries;* Millikan, Robert A.: *Evolution in Science and Religion;* Morgan, Thomas Hunt: *The Theory of the Gene;* Peake, Harold and Fleure, Herbert John: *The Corridors of Time, I–V;* Robbins, Wm. Jacob and Samuel Brody, et al: *Growth;* Sumner, W. G. and Keller, A. G.: *The Science of Society;* Wilm, E. C.: *The Theories of Instinct;* Wilson, Edmund B.: *The Physical Basis of Life.*

The Library also desires to acknowledge the gift of two hundred reprints from Dr. H. McE. Knower; papers from Dr. Fauré-Fremiet, which include the works of Balbianai, Henneguy, Thelohon, Lecauller, and Loyez; and a collection of 131 books from Mr. Ware Cattell; also a collection of reprints on Tunicates and Ascidians, from Dr. Harold S. Colton.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I beg to submit herewith a report of the forty-second session of the Marine Biological Laboratory for the year 1929.

1. *Attendance.* An inspection of the Tabular View of Attendance for the years 1925 to 1929, inclusive, on page 33 will show that since

1927, when crowding of the laboratory buildings and particularly of the Mess during the middle of the summer first became a serious problem, there have been no great changes in the total attendance, the figures for 1927, 1928 and 1929 for investigators and students together being respectively, 434, 454 and 444. It may be noted, however, that although the total for 1929 was slightly less than that for 1928, there was at the same time an increase of 6 in the number of investigators over the previous high record of 323 in 1928. The experience of the past three years indicates that with the present facilities of the Laboratory a materially increased registration will be impossible in the future without an extension of the active season on one or both sides of the present crowded period covering chiefly the months of July and August. How to bring about such an extension is a problem the solution of which will call for the coöperation of all of the workers associated with the Laboratory.

While the total attendance for 1929 was approximately the same as that for 1928, a considerable improvement in the distribution of the attendance was brought about during the year covered by this report by a re-arrangement of the courses of instruction in such a way as to spread them over a period of twelve weeks instead of six as in the past. In 1929 the courses in Botany, Embryology and Physiology were given between June 19 and July 30 inclusive and those in Invertebrate Zoölogy and Protozoölogy between August 1 and September 11, inclusive. Not only did this arrangement reduce the number of students in attendance at any one time during the crowded season, but it tended to emphasize to members of the teaching staffs and to others the desirability of making August 1 a point of division of the season for those unable to be in attendance during the entire summer. An additional advantage of the new arrangement was the freeing of one of the large classrooms and its conversion into nine private laboratories of a desirable type not previously available for investigators.

The results of this change are most strikingly shown by the following figures for attendance at the Mess on selected days for 1928 and 1929.

		1928	1929
May	30	0	0
June	10	63	73
"	20	157	228
"	30	419	304
July	10	484	386
"	20	470	387
"	30	444	386

August	10	335	431
"	20	323	401
"	30	265	357
September	10	124	234
"	20	14	68
"	30	0	0

It will be noted that the sharp peak which characterized the Mess attendance for 1928 and to a somewhat lesser extent that for previous years is greatly flattened in 1929. It may be mentioned that the reduction of more than 11 per cent in the maximum daily attendance (438 on August 7 in 1929 as compared with 495 on July 14, 1928) was accomplished in spite of an increase of 7 per cent in the total number of meals served during the season (33,633 in 1929 as compared with 31,555 in 1928). Furthermore, in 1929 the length of the period during which more than 400 persons were served daily was only 22 days, while in 1928 it was 40 days. It is believed that some slight disadvantages of the new arrangement of the courses are more than compensated for by the reduction in crowding indicated by the above figures.

That the new arrangement of the courses was also not without an effect on the distribution of the attendance by investigators is shown by the following figures for four months each of the three years, 1927, 1928 and 1929, which indicate very clearly a flattening and a spreading of the undesirable peak of attendance for the year last mentioned:

		1927	1928	1929
May	30	7	15	9
June	10	50	64	55
"	20	114	140	139
"	30	212	240	197
July	10	247	281	238
"	20	247	282	242
"	30	245	272	249
August	10	234	250	256
"	20	208	226	243
"	30	168	183	220
September	10	110	112	157
"	20	50	43	59
"	30	12	14	14

2. *The Report of the Treasurer.* This report shows an increase in the total assets of the Laboratory from $2,451,630.65 in 1928 to $2,660,478.82 in 1929, the greater part of this increase being due to the addition to the endowment of the Laboratory of a generous gift of $200,000 for the support of the library, from the General Edu-

cation Board. The total income for the year was slightly less than that for 1928, namely, $200,408.91 as compared with $202,825.09; but, as the expenditures decreased to an even greater extent, the excess of expenditures over income, after allowing for depreciation, was $855.33 in 1929, as compared with $2,715.71 in 1928. During the past year the mortgage obligations of the Laboratory were reduced by $4,500, leaving an amount still outstanding of $27,000. The steady progress made during the past five years in the payment of mortgages is indicated by the following figures representing the amounts so owed at the close of each of the years, 1925 to 1929, inclusive:

1925	$54,030.01
1926	44,500.00
1927	42,500.00
1928	31,500.00
1929	27,000.00

3. *The Report of the Librarian.* The past year has shown a continuation of the rapid and substantial gains in the usefulness of the library made possible by the appropriation of $50,000 by the General Education Board in 1926. This appropriation has been paid in unequal annual installments over the past four years as follows: 1926, $10,000; 1927, $15,000; 1928, $15,000; and 1929, $10,000. With the completion of the gift in 1929 it becomes of interest to tabulate the growth of the library during the period in question.

	1925	1926	1927	1928	1929
Serials received currently	500	628	764	874	985
Total number of bound volumes	15,000	18,200	22,800	26,500	28,300
Reprints	25,000	38,000	43,000	51,000	59,000

It will be noted that the numbers of serials received currently and of bound volumes (mostly serials) have almost doubled since 1925, while the number of reprints has considerably more than doubled. So satisfactory has been the progress of the library under the favorable conditions created by the generosity of the General Education Board that the new gift from this body of $200,000, noted above, the income from which will permit a permanent continuation of the remarkable progress of the past four years, is particularly gratifying.

4. *Change in the By-Laws.* At the 1928 meeting of the Board of Trustees the Executive Committee was instructed to make the matter of the constitution of the Board of Trustees a special order of business and after a thorough consideration of the matter it reported as follows at the regular meeting held on August 13, 1929.

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"The Board of Trustees is practically a self-perpetuating body comprising men who have been Trustees for many years and whose continued interest and counsel are essential for the continued well-being of the Laboratory. The present policy of re-nominating the eight Trustees whose terms expire each year gives little opportunity, however, for seeding into the Board young men who are to carry on the traditions and management of the Laboratory. Different methods were considered for the relief of this situation and the Executive Committee recommends one such method which is embodied in the amendment to the by-laws as stated below. By this method the number of Trustees is somewhat increased, not, however, by increasing the number in each class, but by creating a new group of counsellors known as Trustees Emeriti. Such Trustees shall have the same rights as other Trustees. Any Trustee upon his seventieth birthday will become automatically a candidate for election by the Corporation at the next annual meeting as Trustee Emeritus for life. By filling the vacancies arising in this way young men can be introduced into the Board gradually, and at the same time we can honor the elder statesmen by electing them to this privileged group.

"We recommend, therefore, the following by-law as a substitute for paragraph I of the present By-laws:

"'The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory in Woods Hole, Massachusetts, at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years and in addition there shall be two groups of Trustees as follows: (a) Trustees *ex officio*, who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (b) Trustees Emeriti, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeriti shall each have the same right to vote as the regular Trustees.

"'The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.'"

Upon motion duly made and seconded and after discussion during which it was brought out that similar rights and privileges would be shared by the existing Trustees Emeriti, it was unanimously voted to approve and adopt as a substitute for Article I of the current By-laws the form as recommended by the Executive Committee.

5. The Board of Trustees. In accordance with the change in the By-laws adopted by the Trustees, it was voted by the Corporation at its regular annual meeting to honor by election as Trustees Emeriti C. R. Crane, H. H. Donaldson, W. B. Scott and E. B. Wilson. At the same meeting H. C. Bradley, R. Chambers, E. N. Harvey, and A. C. Redfield were elected to fill the vacancies thus created, while I. F. Lewis, R. S. Lillie, E. P. Lyon, C. E. McClung, T. H. Morgan, and D. H. Tennent of the Class of 1929 were re-elected as members of the Class of 1933.

6. *The Biological Bulletin.* In the Report of the Director for 1928 the enlargement of the Editorial Board of the Biological Bulletin by the election of six new members was mentioned. During 1929 Dr. A. C. Redfield, Assistant Professor of Physiology in the Harvard Medical School, was chosen as Managing Editor to succeed Dr. C. R. Moore, whose valuable services in this capacity since 1927 will be remembered with gratitude by all persons connected with the Laboratory. Arrangements were made for Dr. Redfield's management of the journal to begin with the first number of Volume LVIII, appearing early in 1930.

7. *International Physiological Congress.* The summer of 1929 was a memorable one because of the visits to the Laboratory for periods ranging from a few hours to several months of most of the foreign physiologists who attended the XIIIth International Physiological Congress held in Boston in August of that year. A part of the entertainment arranged for the foreign members of the Congress was a visit to the Marine Biological Laboratory on Saturday, August 24, immediately after the conclusion of the Boston meetings. With the kind assistance of a number of the summer residents of Woods Hole, the Laboratory was able to act as host on that occasion to between five and six hundred physiologists and their wives. The local Committee, of which W. E. Garrey was Chairman and Selig Hecht Secretary, and of which the other members were: Mrs. J. Malcolm Forbes, E. N. Harvey, F. R. Lillie, M. H. Jacobs, Mrs. E. B. Meigs, A. N. Richards and Mrs. J. P. Warbasse, arranged the following program for the day:

12:30 to 1:30 P.M.	Arrival and registration of guests followed by lunch at the Mess.
2:00 to 6:00 P.M.	(a) Demonstration of the scientific activities of the Laboratory and of the U. S. Bureau of Fisheries.

(b) Collecting trips on the Cayadetta.
(c) Sailing trips.
(d) Bathing.
(e) Carriage drives on Naushon Island.
(f) Motion pictures of biological interest.
(g) Teas at the M. B. L. Club House, the U. S. Bureau of Fisheries and on Naushon Island.
6:30 P.M. Clam-bake at Gladheim, the residence of Dr. and Mrs. J. P. Warbasse, Penzance Point.

Most of the visitors departed on the night boat for New York; but after a stay of some days in that city, and in some cases after further travel in the United States and Canada, a considerable number returned to Woods Hole, where they were the guests of the Laboratory for further periods ranging from a few days to several weeks. In addition to these visitors, the Laboratory also had the pleasure of entertaining for varying lengths of time before the opening of the Congress a number of specially invited foreign guests whose presence in Woods Hole contributed much to the scientific and the social life of the community.

8. *Lectures.* The year 1929 was also remarkable for both the number and the importance of the evening lectures delivered during the summer. This memorable season was made possible by the kindness of the distinguished foreign visitors who consented to speak on various aspects of their own important work. During the period between June 21 and September 13, sixteen lectures in all were delivered, twelve of them by visiting foreign scientists, representing eight European countries. An examination of the list of lecturers on page 34 will show a collection of distinguished names which it will be very difficult in the future to equal in any single season.

Of the lectures given in 1929, two were special memorial lectures. These were: The Eighth William Thompson Sedgwick Memorial Lecture, delivered on August 16 by Dr. Torsten Thunberg, Professor of Physiology, University of Lund, Sweden, and the First Reynold A. Spaeth Memorial Lecture, delivered on September 9 by Dr. Rudolf Höber, Professor of Physiology, University of Kiel, Germany.

In addition to the more formal lectures already mentioned, there were also held 9 meetings for the presentation and informal discussion of shorter scientific papers. The total number of papers presented at these meetings was 29; their titles are given on page 35. Since meetings of this type appear to fill a real need of the Laboratory, it is planned to continue them in future years.

9. *Courses.* Under the heading of *Attendance* there has already been mentioned the change in the dates between which the various

courses were held in 1929 and the effects of this change in the reduction of crowding. It was anticipated that the new arrangement might result in a considerable temporary decrease in the number of students in attendance, but the actual decrease proved to be much smaller than was anticipated and was in part attributable to factors other than the times at which the courses were held. On the whole, the new plan was so successful that with certain minor modifications which the experience of the past year has shown to be desirable, it will be continued in 1930.

One change in personnel occurred in 1929 in connection with the direction of the courses, Dr. M. H. Jacobs retiring from the position as head of the Physiology Course which he had held since 1922 and Dr. W. R. Amberson being chosen to take his place, beginning in 1930. There should also be mentioned the very successful direction during 1929 of the Embryology Course by Dr. Charles Packard during the temporary absence of its Head, Dr. H. B. Goodrich.

10. *Gifts.* In addition to the important appropriation received from the General Education Board and already mentioned in the discussion of the reports of the Treasurer and of the Librarian, the following additional gifts are gratefully acknowledged: from Dr. Frank R. Lillie, $1,950 for beautifying the grounds of the Laboratory; from Professor Henri Fredericq, $200 for scholarships in Physiology, to be awarded at the discretion of the Executive Committee; and from the "Collecting Net," through Mr. Ware Cattell, $500 for scholarships to be awarded by a committee consisting of Doctors E. G. Conklin, W. E. Garrey and L. L. Woodruff. Further acknowledgment is also made, with the thanks of the Laboratory, of gifts to the library, already noted in the Report of the Librarian, from Dr. H. McE. Knower, Dr. E. Fauré-Fremiet, Mr. Ware Cattell and Dr. H. S. Colton.

There are appended as parts of this report:

1. The Staff, 1929.
2. Investigators and Students, 1929.
3. A Tabular View of Attendance, 1925–1929.
4. Subscribing and Coöperating Institutions, 1929.
5. Evening Lectures, 1929.
6. Shorter Scientific Papers, 1929.
7. Members of the Corporation, August, 1929.

1. THE STAFF, 1929

Merkel H. Jacobs, *Director,* Professor of General Physiology, University of Pennsylvania.
Associate Director: ——

I. INVESTIGATION

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
E. G. Conklin, Professor of Zoölogy, Princeton University.
Caswell Grave, Professor of Zoölogy, Washington University.
H. S. Jennings, Professor of Zoölogy, Johns Hopkins University.
Frank R. Lillie, Professor of Embryology, The University of Chicago.
C. E. McClung, Professor of Zoölogy, University of Pennsylvania.
S. O. Mast, Professor of Zoölogy, Johns Hopkins University.
T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.
G. H. Parker, Professor of Zoölogy, Harvard University.
E. B. Wilson, Professor of Zoölogy, Columbia University.
Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. INSTRUCTION

J. A. Dawson, Instructor in Zoölogy, Harvard University.
Rudolf Bennitt, Associate Professor of Zoölogy, University of Missouri.
T. H. Bissonnette, Professor of Biology, Trinity College.
E. C. Cole, Associate Professor of Biology, Williams College.
Madeleine P. Grant, Assistant Professor of Zoölogy, Mount Holyoke College.
E. A. Martin, Assistant Professor of Zoölogy, College of the City of New York.
O. E. Nelsen, Instructor in Zoölogy, University of Pennsylvania.
A. E. Severinghaus, Instructor in Zoölogy, Columbia University.

PROTOZOÖLOGY

I. INVESTIGATION

(*See Zoölogy*)

II. INSTRUCTION

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
Mary Stuart MacDougall, Professor of Zoölogy, Agnes Scott College.
W. B. Unger, Assistant Professor of Zoölogy, Dartmouth College.

EMBRYOLOGY

I. INVESTIGATION

(*See Zoölogy*)

II. INSTRUCTION

Hubert B. Goodrich, Professor of Biology, Wesleyan University.
Benjamin H. Grave, Professor of Biology, De Pauw University.
Leigh Hoadley, Assistant Professor of Zoölogy, Harvard University.

Charles Packard, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.
Harold H. Plough, Professor of Biology, Amherst College.
Charles G. Rogers, Professor of Comparative Physiology, Oberlin College.

PHYSIOLOGY

I. INVESTIGATION

Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.
Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.
Ralph S. Lillie, Professor of General Physiology, The University of Chicago.
Albert P. Mathews, Professor of Biochemistry, The University of Cincinnati.

II. INSTRUCTION

Merkel H. Jacobs, Professor of General Physiology, University of Pennsylvania.
William R. Amberson, Assistant Professor of Physiology, University of Pennsylvania.
Edwin J. Cohn, Assistant Professor of Physical Chemistry, Harvard University.
E. Newton Harvey, Professor of Physiology, Princeton University.
Charlotte Haywood, Assistant Professor of Physiology, Vassar College.
Selig Hecht, Associate Professor of Biophysics, Columbia University.
Leonor Michaelis, Resident Lecturer in Medical Research, Johns Hopkins University.
Alfred C. Redfield, Assistant Professor of Physiology, Harvard University.

BOTANY

I. INVESTIGATION

B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.
C. E. Allen, Professor of Botany, University of Wisconsin.
S. C. Brooks, Professor of Zoölogy, University of California.
Ivey F. Lewis, Professor of Biology, University of Virginia.
Wm. J. Robbins, Professor of Botany, University of Missouri.

II. INSTRUCTION

William Randolph Taylor, Professor of Botany, University of Pennsylvania.
James P. Poole, Professor of Evolution, Dartmouth College.
Ethel M. Poulton, Leessel Fellow, Yale University.

LIBRARY

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.), Librarian.
Deborah Lawrence, Secretary.
Hester Ann Bradbury, Lillian F. Briggs, Mary A. Rohan, Assistants.

BIOLOGICAL LABORA WOODS HOLE LIBRARY

CHEMICAL SUPPLIES

Oliver S. Strong, Professor of Neurology, and Neuro-Histology, Columbia University, *Chemist.*

APPARATUS ROOM

Samuel E. Pond, Assistant Professor of Physiology, Medical School, University of Pennsylvania, *Custodian of Apparatus.*

SUPPLY DEPARTMENT

George M. Gray, Curator.
John J. Veeder, Captain.
E. M. Lewis, Engineer.
A. W. Leathers, Head of Shipping Department.
A. M. Hilton, Collector.
J. McInnis, Collector.
Milton B. Gray, Collector.

F. M. MacNaught, Business Manager.
Herbert A. Hilton, Superintendent of Buildings and Grounds.
Thomas Larkin, Superintendent of Mechanical Department.
Lester F. Boss, Mechanician.
William Hemenway, Carpenter.
J. D. Graham, Glass-blowing Service.
A. R. Apgar, Photographic Service.
Arnold H. Bisco, Storekeeper and Head Janitor.

2. INVESTIGATORS AND STUDENTS, 1929

Independent Investigators

Addison, William H. F., Professor of Normal Histology and Embryology, University of Pennsylvania.
Allen, C. E., Chairman, Division of Biology and Agriculture, National Research Council.
Amberson, William R., Assistant Professor of Physiology, University of Pennsylvania.
Armstrong, Philip, Instructor in Anatomy, Cornell University Medical College.
Asher, Leon, Director, Physiological Institution, Berne, Switzerland.
Asher, Mrs. Leon, Physiological Institution, Berne, Switzerland.
Austin, Mary L., Instructor in Zoölogy, Wellesley College.
Barcroft, Joseph, Professor of Physiology, Cambridge University, England.
Barron, E. S. Guzman, Instructor in Medicine, Johns Hopkins University.
Barth, L. G., Graduate Student, University of Chicago.
Belar, K. J., Kaiser Wilhelm Institut für Biologie, Berlin-Dahlem, Germany.
Bennitt, Rudolf, Associate Professor of Zoölogy, University of Missouri.
Bethe, A., Professor, Institut f. Animalische Physiologie, Frankfurt, Germany.
Bigelow, Robert P., Professor of Zoölogy, Massachusetts Institute of Technology.
Bissonnette, T. Hume, Professor of Biology, Trinity College.
Blake, Charles H., Instructor, Massachusetts Institute of Technology.
Blanchard, Kenneth C., Assistant Professor of Biochemistry, Washington Square College, New York University.
Blumenthal, Reuben, Graduate Student, University of Pennsylvania.

BORODIN, DMITRY, Pasadena, California.

BOWEN, ROBERT H., Professor of Zoölogy, Columbia University.

BOYD, GEORGE H., Professor of Zoölogy, University of Georgia.

BRADLEY, HAROLD C., Professor of Physiological Chemistry, University of Wisconsin.

BREBNER, WILLIAM B., Washington University Medical School.

BREITENBECHER, J. K., Research Assistant and Lecturer in Zoölogy, McGill University.

BRIDGES, CALVIN B., Research Assistant, Carnegie Institution of Washington.

BRINLEY, FLOYD J., Professor of Physiology, Battle Creek College.

BROOKS, MATILDA M., Research Associate in Biology, University of California.

BUDINGTON, ROBERT A., Professor of Zoölogy, Oberlin College.

BUISSET, SIMONE, C. R. B. Fellow, University of Pennsylvania.

BUTCHER, EARL, Assistant Professor, Hamilton College.

BUTLER, ELMER G., Assistant Professor of Biology, Princeton University.

CALKINS, GARY N., Executive Officer, Department of Zoölogy, Columbia University.

CANNAN, ROBERT K., Senior Lecturer, University College, London, England.

CARMICHAEL, EMMETT B., Associate Professor and Head of Department of Physiological Chemistry, School of Medicine, University of Alabama.

CAROTHERS, E. ELEANOR, Lecturer in Zoölogy, University of Pennsylvania.

CARROLL, PAUL L., Instructor in Biology, Saint Louis University.

CARVER, G. L., Professor of Biology, Mercer University.

CATTELL, MCKEEN, Assistant Professor of Physiology, Cornell University Medical College.

CATTELL, WARE, Research Fellow in Biophysics, Memorial Hospital.

CHAMBERS, ROBERT, Research Professor of Biology and Chairman of Department of Biology, Washington Square College, New York University.

CHEMIN, EMILE A., Professor, Lycée, Buffon, Paris, France.

CHENEY, RALPH H., Assistant Professor of Biology, Washington Square College, New York University.

CHIDESTER, F. E., Professor of Zoölogy, West Virginia University.

CHOUKE, K. S., Instructor in Anatomy, University of Colorado, School of Medicine.

CHRISTIE, JESSE R., Associate Nematologist, United States Department of Agriculture.

CLARK, ELEANOR L., Investigator, University of Pennsylvania.

CLARK, ELIOT R., Professor of Anatomy, University of Pennsylvania.

CLOWES, G. H. A., Director, The Lilly Research Laboratory, Eli Lilly & Co.

COBB, N. A., Nematologist, United States Department of Agriculture.

COE, WESLEY R., Professor of Biology, Yale University.

COHEN, BARNETT, Associate Professor of Physiological Chemistry, Johns Hopkins Medical School.

COLE, ELBERT C., Associate Professor of Biology, Williams College.

CONKLIN, EDWIN G., Research Professor of Biology, Princeton University.

COPELAND, MANTON, Professor of Biology, Bowdoin College.

COWDRY, E. V., Professor of Cytology, Washington University Medical School.

COWLES, R. P., Associate Professor of Zoölogy, Johns Hopkins University.

CRABB, E. D., Instructor in Zoölogy, University of Pennsylvania.

CURTIS, W. C., Professor of Zoölogy, University of Missouri.

DAVIS, EVERETT F., Assistant Plant Physiologist, Virginia Agricultural Experiment Station.

DAVIS, FANNY-FERN SMITH, Assistant Plant Physiologist, Virginia Agricultural Experiment Station.

DAVIS, HALLOWELL, Assistant Professor of Physiology, Harvard University Medical School.

Dawson, J. A., Instructor in Invertebrate Zoölogy, Harvard University.
DeCaro, Luigi, Assistant, University of Naples, Naples, Italy.
Dobzhansky, Theodosius, Research Fellow, International Education Board.
Dodds, G. S., Professor of Histology and Embryology, West Virginia University Medical School.
Dolley, Wm. L., Professor of Biology, University of Buffalo.
Donaldson, Henry H., Member, Wistar Institute.
Donaldson, John C., Associate Professor of Anatomy, University of Pittsburgh.
Du Bois, Eugene F., Associate Professor of Medicine, Cornell University Medical College.
Edwards, Dayton J., Associate Professor of Physiology, Cornell University Medical College.
Failla, G., Physicist, Memorial Hospital.
Faulkner, Gwendolene H., Commonwealth Fund, 1 East 57th Street, New York City.
Frank, Otto, Director, Physiological Institute, University of Munich, Germany.
Fauré-Fremiet, E., Professor of Comparative Embryology, Collège de France, Paris, France.
Fredericq, Henri, Professor of Physiology, University of Liége, Belgium.
Fremont-Smith, Frank, Assistant Professor of Neuropathology, Harvard University Medical School.
Fry, Henry J., Associate Professor of Biology, Washington Square College. New York University.
Gardiner, Mary S., Instructor, Bryn Mawr College.
Garrey, W. E., Professor of Physiology, Vanderbilt University Medical School.
Gelfan, Samuel, Research Fellow, University of Chicago.
Glaser, Otto, Professor of Biology, Amherst College.
Goldforb, A. J., Professor of Biology, College of the City of New York.
Gordon, Myron, Hechscher Research Assistant in Animal Biology, Cornell University.
Grant, Madeleine, Assistant Professor, Mount Holyoke College.
Grave, B. H., Professor of Zoölogy, DePauw University.
Grave, Caswell, Professor of Zoölogy, Washington University.
Green, Arda Alden, National Research Fellow in Medicine, Harvard University Medical School.
Grodzinski, Z., Professor, University of Krakow, Poland.
Grundfest, Harry, Gottsberger Fellow, Columbia University.
Hahn, A., Assistant Professor, Physiological Institute, Munich, Germany.
Hartline, H. K., National Research Fellow in Medicine, Johns Hopkins University.
Harvey, Ethel B., Instructor, Washington Square College, New York University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Hausman, Sibyl A., Laboratory Assistant in Zoölogy, Wellesley College.
Haywood, Charlotte, Assistant Professor of Physiology, Vassar College.
Hecht, Selig, Professor of Biophysics, Columbia University.
Heilbrunn, L. V., Washington Square College, New York University.
Hertig, Arthur T., Medical Student, Harvard University Medical School.
Hibbard, Hope, Assistant Professor, Oberlin College.
Hill, Samuel E., Fellow in Zoölogy of National Research Council, Princeton University.
Hinrichs, Marie A., Research Associate in Physiology, University of Chicago.
Hoadley, Leigh, Assistant Professor, Harvard University.
Höber, Josephine, Physiologische Institut der Universitat, Kiel, Germany.
Höber, Rudolf, Director der Physiologische Institut der Universitat Kiel, Germany.

Holmes, Gladys E., Instructor, Western College for Women.
Howard, Harvey J., Professor of Ophthalmology, Washington University Medical School.
Howe, H. E., Editor, Industrial Engineering Chemistry.
Howland, Ruth B., Associate Professor, Washington Square College, New York University.
Huettner, Alfred F., Associate Professor, Washington Square College, New York University.
Huff, Clay G., Fellow in Tropical Medicine, Harvard University Medical School.
Irwin, Marian, Associate in the Division of General Physiology, Rockefeller Institute for Medical Research.
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.
Jennison, Marshall W., Assistant in Biology, Massachusetts Institute of Technology.
Johlin, J. M., Associate Professor of Biochemistry, Vanderbilt University Medical School.
Jorpes, J., Assistant Professor of Biochemistry, The Caroline Institute, Stockholm, Sweden.
Keefe, Anselm M., Rector and Professor of Biology, St. Norbert College.
Kestner, Otto, Professor of Physiology, University of Hamburg, Germany.
Kindred, James E., Associate Professor of Histology and Embryology, University of Virginia.
Kitchen, S. F., Special Member, International Health Division, Rockefeller Foundation.
Knowlton, Frank P., Professor of Physiology, Syracuse University, College of Medicine.
Knower, H. McE., Woods Hole, Massachusetts.
Krogh, August, Professor of Zoöphysiology, Laboratory of Zoöphysiology, Copenhagen University, Copenhagen, Denmark.
Krumbhaar, Edward B., Professor of Pathology, University of Pennsylvania.
Lackey, J. B., Professor of Biology, Southwestern University.
Lancefield, Donald E., Associate Professor in Zoölogy, Columbia University.
Lancefield, Rebecca C., Assistant, Rockefeller Institute for Medical Research.
Lange, Mathilde, Professor of Zoölogy, Wheaton College.
Lapicque, Louis, Directeur, Laboratoire de Physiologie Générale, Sorbonne, Paris, France.
Lapicque, Marcelle, Directeur Adjoint de Laboratoire de Physiologie Générale, Sorbonne, Paris, France.
Lillie, Frank R., Chairman of the Department of Zoölogy, University of Chicago.
Lillie, Ralph S., Professor of General Physiology, University of Chicago.
Loeb, Leo, Professor of Pathology, Washington University Medical School.
Looper, James B., Associate Professor of Histology and Embryology, University of Mississippi.
Lucké, Balduin, Associate Professor of Pathology, University of Pennsylvania.
Lynch, Ruth Stocking, Instructor in Graduate Zoölogy, The Johns Hopkins University.
McClung, Clarence E., Director, Zoölogical Laboratory, University of Pennsylvania.
McCutcheon, Morton, Assistant Professor of Pathology, University of Pennsylvania.
MacDougall, Mary S., Head of Biology Department, Agnes Scott College.
McGlone, Bartgis, Instructor, University of Pennsylvania.
McGoun, Ralph C., Jr., Instructor in Biology, Amherst College.
Macklin, Charles C., Professor of Anatomy, University of Western Ontario.
Marsland, Douglas A., Assistant Professor of Biology, Washington Square College, New York University.

Martin, Earl A., Assistant Professor, The College of the City of New York.
Mavor, James W., Professor of Biology, Union College.
Menkin, Valy, Fellow in Medicine, Henry Phipps Institute, University of Pennsylvania.
Metz, Charles W., Member of Staff, Department of Genetics, Carnegie Institution of Washington.
Michaelis, L., Resident Lecturer, Johns Hopkins University.
Mills, Clarence A., Associate Professor of Internal Medicine, University of Cincinnati, College of Medicine.
Morgan, Lilian V., California Institute of Technology.
Morgan, T. H., Director of the Biological Laboratory, California Institute of Technology.
Morgulis, Sergius, Professor of Biochemistry, University of Nebraska, College of Medicine.
Morris, Helen S., Graduate Student, Columbia University.
Mulder, Arthur G., Assistant Professor of Physiology, University of Tennessee.
Murray, Margaret R., Associate Professor of Physiology, Florida State College for Women.
Nabrit, Samuel M., Professor of Biology, Morehouse College.
Needham, Joseph, Senior Demonstrator in Biochemistry, Biochemical Laboratory, Cambridge University, England.
Nelsen, Olin E., Instructor in Zoölogy, University of Pennsylvania.
Nonidez, José F., Assistant Professor of Anatomy, Cornell University Medical College.
Nordmann, Martin, Privatdozent of Pathology, University of Kōln, Kōln, Germany.
Osterhout, W. J. V., Member, Rockefeller Institute for Medical Research.
Packard, Charles, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.
Parker, George H., Director, Harvard Zoölogical Laboratory, Harvard University.
Parmenter, Charles L., Associate Professor, University of Pennsylvania.
Payne, Nellie M. C., Scientific Staff, Biological Abstracts, University of Pennsylvania.
Phillips, Paul L., Assistant Instructor in Anatomy, Cornell University Medical College.
Philpott, Charles H., Harris Teachers College.
Plough, Harold H., Professor of Biology, Amherst College.
Plowe, Janet Q., Graduate Student, University of Pennsylvania.
Pollister, Arthur W., Instructor in Zoölogy, Columbia University.
Pond, Samuel E., Assistant Professor of Physiology, University of Pennsylvania.
Poole, James P., Professor of Evolution, Dartmouth College.
Potter, Truman S., Seymour Coman Fellow, University of Chicago.
Poulton, Ethel M., Leessel Fellow, Yale University.
Ratzlaff, Abe K., Assistant Instructor, University of Kansas.
Redfield, Helen, Assistant in Biology, Washington Square College, New York University.
Reese, Albert M., Professor of Zoölogy, West Virginia University.
Rehberg, P. B., Assistant, Zoöphysiological Laboratory, University of Copenhagen, Denmark.
de Rényi, George St., Associate Professor of Anatomy, University of Pennsylvania.
Reznikoff, Paul, Instructor in Medicine, Cornell University Medical College.
Richards, Alfred N., Professor of Pharmacology, University of Pennsylvania.
Richards, Oscar W., Assistant Professor of Biology, Clark University.

Rogers, Charles G., Professor of Comparative Physiology, Oberlin College.
Rugh, Roberts, Assistant in Zoölogy, Columbia University.
Rylant, Pierre, Professor, Universitat de Bruxelles, Belgium.
Samojloff, A., Professor of Physiology, University of Kazau, Russia.
Sayles, Leonard P., Instructor in Zoölogy, Tufts College.
Schultz, Jack, National Research Fellow, Columbia University.
Schumann, Margaret, Technician, Microscopic Anatomy, University of Kansas.
Schwind, Joseph L., Instructor in Anatomy, Cornell University Medical College.
Sen, Basiswar, Director, Vivekananda Laboratory, Calcutta, India.
Sereni, Enrico, Capo del Reparto di Fisiologia, Stazione Zoologica, Napoli.
Severinghaus, Aura E., Instructor in Anatomy, College of Physicians and Surgeons.
Shanklin, William M., Adjunct Professor of Histology, American University of Beirut.
Shull, A. Franklin, Professor of Zoölogy, University of Michigan.
Shumway, Waldo, Professor of Zoölogy, University of Illinois.
Smith, Helen B., Graduate Student, Johns Hopkins University.
Snyder, Charles, Professor of Experimental Physiology, Johns Hopkins University Medical School.
Sokoloff, Boris, Professor of Experimental Medicine, University of Prague, Czechoslovakia.
Speidel, Carl C., Associate Professor of Anatomy, University of Virginia.
Steinbach, H. B., Demonstrator in Physiology, Brown University.
Stern, Lina, Professor of Physiology, University and Physiological Institute of Moscow, Russia.
Strong, Oliver S., Professor of Neurology and Neuro-Histology, Columbia University.
Stunkard, H. W., Professor of Biology, New York University.
Sturtevant, A. H., Professor of Genetics, California Institute of Technology.
Sumwalt, Margaret, Associate in Physiology and Pharmacology, Women's Medical College.
Sun, T. P., Student, University of Pennsylvania.
Swett, F. H., Associate Professor of Anatomy, Vanderbilt University Medical School.
Taylor, Ivon R., Instructor in Physiology, Brown University.
Taylor, William R., Professor of Botany, University of Pennsylvania.
Tennent, David H., Professor of Biology, Bryn Mawr College
Turner, Abby H., Professor of Physiology, Mount Holyoke College.
Tyler, Albert, Department of Biology, California Institute of Technology.
Uhlenhuth, Eduard, Associate Professor in Anatomy, University of Maryland Medical School.
Unger, W. Byers, Assistant Professor of Zoölogy, Dartmouth College.
Van Slyke, E., Instructor, University of Pittsburgh.
Verzár, Frederic, Professor, University of Debrescsen, Hungary.
Vicari, Emilia, Research Assistant, Cornell University Medical College.
Wang, Chi Che, Head of Department of Chemistry, Nelson Morris Memorial Institute for Medical Research.
Warren, Howard C., Stuart Professor of Psychology, Chairman of Department, Princeton University.
Whiting, Anna R., Professor, Pennsylvania College for Women.
Whiting, P. W., Associate Professor of Zoölogy, University of Pittsburgh.
Wieman, H. L., Professor of Zoölogy, University of Cincinnati.
Willey, Charles H., Instructor in Biology, New York University.
Williams, S. Culver, Instructor, University of Pennsylvania.

Wilson, Edmund B., DaCosta Professor of Zoölogy, Emeritus, Columbia University.
Wilson, May G., Instructor, Cornell University Medical College.
Winterstein, Hans, Director, Physiological Institute of the University of Breslau, Germany.
Wodehouse, R. P., 45 Bridge Drive, Yonkers, New York.
Wolf, E. Alfred, Assistant Professor, University of Pittsburgh.
Wolff, William A., Research Assistant in Pharmacology, University of Pennsylvania.
Woodruff, L. L., Professor of Protozoölogy, Yale University.
Wormall, Arthur, Lecturer in Biochemistry, The School of Medicine, Leeds, England.
Wrede, Fritz, Professor of Physiology, Medical Faculty, University of Greifswald, Germany.
Young, Roger Arliner, Assistant Professor, Howard University.

Beginning Investigators

Abell, Richard G., Instructor in Zoölogy, University of Pennsylvania.
Adams, Theodore G., Assistant Instructor, Columbia University.
Bailey, Sarah W., Student, Radcliffe College.
Baron, A. L., Assistant in Biology, Washington Square College, New York University.
Benkert, Joseph M., Graduate Assistant, University of Pittsburgh.
Bennett, Harry J., Assistant in Zoölogy, Illinois State University.
Biddle, Russell L., Teaching Fellow, California Institute of Technology.
Bostian, Carey, Graduate Assistant, University of Pittsburgh.
Bostian, Neita C., Research Assistant, University of Pittsburgh.
Bowling, Rachel, Assistant in Zoölogy, Columbia University.
Bowman, Paul W., Instructor in Botany, George Washington University.
Brooks, Chandler M., Graduate Assistant, Princeton University.
Burkhardt, Ludwig, Anatomisches Institut, Würzburg, Germany.
Carson, Rachel L., Pennsylvania College for Women.
Cashdollar, Helen, University of Pittsburgh.
Chalmers, A. Elizabeth, University of Pittsburgh.
Charles, Donald R., Graduate Assistant, University of Pittsburgh.
Chase, Aurin M., Laboratory Assistant, Amherst College.
Chesley, Leon C., Fellow in Zoölogy, Duke University.
Cline, Elsie, Graduate Student, The Johns Hopkins University.
Comegys, Margaret B., Assistant Professor, Long Island University.
Croasdale, Hannah T., University of Pennsylvania.
Curtis, Mary E., Graduate Student, Yale University.
Deitrick, John E., Student, Princeton University.
Draper, John W., Medical Student, Cornell University Medical College.
Ehrlich, William, Medical Student, Washington University Medical School.
Fraser, Doris A., Assistant in Histology, University of Pennsylvania.
Frew, Priscilla E., Instructor, Washington Square College, New York University.
Genther, Ida T., Assistant in Pathology, Washington University Medical School.
Grannis, Frank C., Professor of Biology, Lincoln Memorial University.
Graubard, Marc A., Assistant in Zoölogy, Columbia University.
Heller, Richard E., Research Student, University of Chicago.
Henderson, Jean T., Assistant in Biology, Washington Square College, New York University.
Hetherington, W. Alford, Teaching Fellow, California Institute of Technology.
Hiyeda, Kentaro, Assistant Professor of Pathology, Manchuria Medical College.
Hook, Sabra J., Assistant in Zoölogy, Barnard College, Columbia University.

Johnston, Janet L., Demonstrator in Biology, Brown University.
Keil, Elsa M., Assistant in Biology, Brown University.
Keosian, John, Assistant in Biology, Washington Square College, New York University.
Kerr, Thomas, Assistant in Biology, Washington Square College, New York University.
Kinney, Elizabeth T., Graduate Student, Columbia University.
McCardle, Ross C., Instructor in Physiology, Temple University.
McCrady, Edward, Jr., Graduate Assistant in Zoölogy, University of Pittsburgh.
McWilliams, Thelma L., Assistant, University of Wisconsin.
Miller, Evelyn H., Graduate Student, University of Pennsylvania.
Monosmith, Helen, Research Assistant, Carnegie Institution.
Moore, Imogene, Graduate Student, Yale University.
Oliphant, J., Baltimore, Maryland.
Parks, Elizabeth K., Graduate Assistant in Zoölogy, Oberlin College.
Parpart, Arthur K., Graduate Student, University of Pennsylvania.
Parpart, Ethel Roberta, Technician, University of Pennsylvania.
Pierson, Bernice F., Assistant, University of Maryland.
Powsner, Louis, Student, Harvard University.
Robert, Nan L., Columbia University.
Root, Walter S., Assistant Instructor, University of Pennsylvania.
Rowlee, Silence, Assistant Professor, Elmira College.
Sayles, E. Duane, Graduate Assistant of Zoölogy, University of Chicago.
Sears, Mary, Student, Radcliffe College.
Sichel, Ferdinand J., Assistant in Biology, Washington Square College, New York University.
Snell, George D., Graduate Student, Bussey Institution, Harvard University.
Steinhardt, Jacinto, Instructor in Biophysics, Columbia University.
Stewart, Dorothy R., Graduate Student, University of Pennsylvania.
TerLouw, Adrian, Graduate Student, Johns Hopkins University.
TeWinkel, Lois E., Assistant in Zoölogy, Barnard College, Columbia University.
Thigpen, Lorna W., Graduate Student, University of Pittsburgh.
Thompson, Guy P., Instructor in Zoölogy, University of Maryland.
Torvik, Magnhild M., Graduate Assistant, University of Pittsburgh.
Verrijp, C. D., University of Leiden, Donders Foundation, Leiden, Netherlands.
Wald, George, Research Assistant, Columbia University.
Whipple, Allen O., Professor of Surgery, Columbia University.
Yamaguchi, Seiji, Assistant Professor, Manchuria Medical College, Manchuria, Japan.

Research Assistants

Armstrong, Eleanor F., Research Assistant, Cornell University Medical College.
Averell, Philip R., Research Assistant, Rockefeller Institute for Medical Research
Bernstein, Alan, Research Assistant, New York University.
Buck, Louise H., Research Assistant, Columbia University.
Carroll, Margaret P., Research Assistant, Harvard University Medical School.
Cotui, Frank, Instructor in Pharmacology, New York University.
Dailey, Mary E., Research Chemist, Harvard University Medical School.
Eisenbrandt, William H., Research Assistant, University of Maryland Medical School.
Friedman, Hilda, Assistant in Pathology, Washington University Medical School.
Greene, Eunice C., Research Fellow in Anatomy, College of Medicine, Syracuse University.
Harnly, Marie L., Research Assistant, Carnegie Institution of Washington.
Keltch, Anna K., Lilly Research Laboratory, Eli Lilly & Co.

Lorberblatt, Isaac, Medical Student, Washington University Medical School.
McDonald, Joseph A., Research Assistant, Washington Square College, New York University.
Sanders, Rosaltha, Technical Assistant, Yale University.
Scarborough, Eliott, Medical Student, University of Alabama.
Schauffler, W. G., Princeton, New Jersey.
Schmuck, M. Louise, Research Assistant, Carnegie Institution of Washington.
Schubert, Maxwell, Rockefeller Institute.
Shlaer, Simon, Research Assistant, Columbia University.
Smith, Mary Doreen, Research Assistant, Memorial Hospital.
Smith, Wilbur A., Medical Student, University of Pennsylvania.
Wallace, Edith M., Artist and Research Assistant, Carnegie Institution of Washington.
Waterman, Allyn J., Harvard University.

Students

BOTANY

Aldinger, Lenore, 157 East 67th Street, New York City.
Coggeshall, Mary, Johns Hopkins University.
Crone, Mary Catherine, Western Reserve University.
Graustein, Jeannette E., Radcliffe College.
Jordon, Phoebe, Smith College.
Morgan, Angie, Teacher of Biology, Paris High School, Cooper, Texas.
Moyer, Lawrence S., Assistant in Botany, University of Pennsylvania.
Murray, Grace L., Science Teacher, University of Rochester.
Rippey, Aaron S., Student, George Washington University.
Thygeson, Christine, Junior Botanist, United States Department of Agriculture.
Tuan, Hsu C., Student, University of Pennsylvania.
Wilson, Sarah E., Goucher College.

EMBRYOLOGY

Bailey, Ralph J., Assistant in Zoölogy, University of Wisconsin.
Behney, William H., Research Fellow, University of Vermont.
Bennett, Harry J., Assistant in Zoölogy, University of Illinois.
Bilstad, Nellie M., Assistant in Zoölogy, University of Wisconsin.
Burr, Barbara, St. Lawrence University.
DeRoo, Grace I., Bryn Mawr College.
DuShane, Graham P., Undergraduate, Wabash College.
Emerson, Henry S., Amherst College.
Feighner, Lena V., Teacher of General Science, Kansas City.
Findlay, Martha S., Graduate Student, Radcliffe College.
Fish, Harold S., Instructor in Biology, Colby College.
Gooding, Alice A., Instructor, Hunter College.
Grubb, Thomas C., Student, Hamilton College.
Hunter, Richard H., Lecturer in Anatomy, Queens' University, Belfast, Ireland.
Koff, Arthur K., Instructor in Pathology, Johns Hopkins University.
Kopenhaver, Mary Elizabeth, Student, Hood College.
Nussmann, Theodora C., Fellow, University of Cincinnati.
Rudnick, Dorothea, Student, University of Chicago.
Schiro, Harold S., Bowdoin College.
Scott, Marie, Assistant Instructor in Zoölogy, University of Kansas.
Scott, Sister Florence Marie, Assistant Professor of Zoölogy, Seton Hill College.
Smith, Ernest V., Student, Wabash College.

Steiner, Matthew M., New York University.
Street, Sibyl F., Assistant, Vassar College.
Walker, Roland, Oberlin College.
Wickenden, James N., Oberlin College.
Wolfe, Mary F., Radcliffe College.
Zimmerman, Elizabeth K., Hood College.

PHYSIOLOGY

Du Bois, Eugene F., Assistant Professor of Medicine, Cornell University Medical College.
Fowler, John Robert, Student, University of Chicago.
Haynes, Florence W., Mount Holyoke College.
Heller, Richard E., Student, University of Chicago.
Henshaw, Paul S., Research Fellow, University of Wisconsin.
Hoffman, Lloyd A., Johns Hopkins Medical School.
Howes, Samuel A., Instructor in Botany, Rhode Island State College.
Jorpes, Johan E., Assistant Professor in Biochemistry, Caroline Institute, Stockholm.
Kerr, Thomas, Assistant in Biology, New York University.
McGavran, Joyce, Instructor in Physiology, College for Women, Western Reserve University.
Millikan, Glenn A., California Institute.
Nitzulescu, Julius J., Assistant, School of Medicine, Rumania.
Packard, Charles E., Instructor, University of Cincinnati.
Sayles, Duane, Graduate Assistant, University of Chicago.
Seelye, Edwin B., Amherst College.
Stuck, Ralph M., Student, Washington University Medical School.
Wright, Charles I., Rochester University.

PROTOZOÖLOGY

Carroll, Paul L., Instructor in Biology, St. Louis University.
Ellis, John M., Head of Biology Department, Junior College of Augusta.
Hausman, Sibyl A., Assistant in Zoölogy, Wellesley College.
Hileman, Clara M., Teacher, High School, Valley Stream, Long Island.
Hiyeda, Kentaro, Assistant Professor of Pathology, Manchuria Medical College.
Hoppe, Marguerite E., Teachers College, Columbia University.
Jones, Edgar P., Instructor, University of Pittsburgh.
Morgan, Angie, Teacher of Biology, Paris High School, Cooper, Texas.
Oltmann, Clara, Student, Columbia University.
Rugh, Roberts, Instructor, Hunter College.
Scott, Sister Florence Marie, Teacher of Biology, Seton Hill College.
Smith, Thomas L., Head of the Department of Biology, The College of the Ozarks.
Stuck, Florence, Student, Columbia University.
Whitehead, Katisue, Graduate Assistant, University of Georgia.
Willis, Alma A., Graduate, Columbia University.

INVERTEBRATE ZOÖLOGY

Ameel, Donald J., Graduate Assistant, University of Michigan.
Bailey, Sarah W., Student, Radcliffe College.
Ballew, Amer M., Instructor, Austin High School, Chicago.
Bell, Leslie M., Assistant in Zoölogy and Embryology, University of Alabama.
Blondel, Dorothy M., Barnard College.

Bradley, Frederick W., Upper Montclair, New Jersey.
Brehme, Katherine S., Barnard College.
Campbell, Malcolm D., Junior Master, Dorchester High School for Boys.
Capps, Harry M., Jr., Student, Amherst College.
Chambers, Joseph C., Jr., Student, Amherst College.
Collier, Cornelia H., Student, Elmira College.
Coonfield, Benjamin R., Austin Teaching Fellow, Harvard University.
Crane, Barbara, Student, Oberlin College.
Cummings, Frances, Student, Albertus Magnus.
Darby, Pauline, Assistant, Washington University.
Finkelstein, Ruth, Johns Hopkins University.
Flammon, Sister M. Muriel, Seton Hill College.
Foster, Aurel O., Assistant in Zoölogy, Wesleyan University.
Frost, Nancy, Acadia University.
Frye, Mary Elizabeth, Pennsylvania College for Women.
Gould, Robert G., Jr., Student, Harvard University.
Haas, Anna L., H. Sophie Newcomb College.
Handford, Stanley W., Graduate Student, Yale University.
Hastings, Alice, Mount Holyoke College.
Hayden, Sherman S., Harvard University.
Hummel, Elsie C., Teacher of Biology, Lasell Seminary.
Keck, Henry E., Assistant in Biology, Ohio University.
Kendall, James I., Student, Bussey Institution.
Kerr, Kathel B., Student, Oberlin College.
Kumpf, Katharine F., Goucher College.
Lammers, Helene N., Assistant in Biology, Agnes Scott College.
LeCates, Carrie E., University of Delaware.
Lloyd, Janet, Smith College.
Loughridge, Gasper A., Teaching Fellow in Botany, Rutgers University.
MacFarland, William E., Student, Acadia University.
Nichols, Rowena, Student, Wellesley College.
Patterson, Martha M., Senior, Wilson College.
Phelps, Austin, Assistant, Yale University.
Plyler, Phyllis V., Goucher College.
Rudnick, Dorothea, Fellow in Zoölogy, University of Chicago.
Scott, John Paul, University of Wyoming.
Sitomer, Mindel, Student, New York University.
Spalding, Alice Dean, Teachers College, Columbia University.
Stauber, Leslie A., Graduate Assistant in Zoölogy, Rutgers University.
Stiles, Karl A., Instructor of Biology, Battle Creek College.
Stoelting, Dorothy E., Student, Butler University.
Stultz, Walter A., Assistant in Zoölogy, Yale University.
Sweeney, Julia D., Senior, Radcliffe College.
Toothill, Martha C., Mount Holyoke College.
Torrey, Theodore W., Student, Harvard University.
Wald, Helena, Student, New York University.
Waller, William H., Student, De Pauw University.
Weston, Florence E., Graduate Student, Tufts College.

3. TABULAR VIEW OF ATTENDANCE

	1925	1926	1927	1928	1929
INVESTIGATORS—Total	207	252	294	323	329
Independent	135	156	209	217	234
Under Instruction	72	84	57	81	71
Research Assistants		12	28	25	24
STUDENTS—Total	132	141	141	133	125
Zoölogy	54	56	57	57	53
Protozoölogy	17	19	17	16	15
Embryology	29	28	32	29	28
Physiology	19	18	19	15	17
Botany	13	20	16	16	12
TOTAL ATTENDANCE	339	393	435	456	454
Less Persons registered as both students and investigators		8	1	2	10
		385	434	454	444
INSTITUTIONS REPRESENTED—Total	112	119	111	111	123
By investigators	74	84	89	80	96
By students	65	60	63	66	64
SCHOOLS AND ACADEMIES REPRESENTED					
By investigators	1	–	1	1	
By students	4	4	4	1	1
FOREIGN INSTITUTIONS REPRESENTED					
By investigators	–	17	15	13	30
By students	–	3	8	8	3

4. SUBSCRIBING AND COOPERATING INSTITUTIONS

Acadia University
Agnes Scott College
Amherst College
Barnard College
Battle Creek College
Bowdoin College
Brown University
Bryn Mawr College
Butler College
C. R. B. Educational Foundation
California Institute of Technology
Carnegie Institution, Cold Spring Harbor
Carnegie Institution of Washington
Chinese Educational Mission
Columbia University
Commonwealth Fund
Cornell University
Cornell University Medical College
De Pauw University
Duke University
Elmira College
General Education Board
Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Howard University
Industrial & Engineering Chemistry, of the American Chemical Society
International Education Board
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly & Co.
Massachusetts Institute of Technology
Memorial Hospital of New York City
Morehouse College
Mount Holyoke College
National Research Council
New York University

Oberlin College
Ohio University
Pennsylvania College for Women
Princeton University
Radcliffe College
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Seton Hill College
Smith College
Sophie Newcomb College
Southwestern
St. Norbert College
Tufts College
Union College
United States Dept. of Agriculture
University of Alabama
University of Buffalo
University of Chicago
University of Cincinnati
University of Delaware
University of Illinois
University of Kansas
University of Maryland Medical School
University of Michigan
University of Missouri
University of Nebraska
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Vermont
University of Virginia
University of Wisconsin
Vanderbilt University Medical School
Vassar College
Wabash College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Western Reserve University
West Virginia University
Wistar Institute of Anatomy and Biology
Women's Medical College of Pennsylvania
Yale University

SCHOLARSHIP TABLES

Ida H. Hyde Scholarship of the University of Kansas.
Lucretia Crocker Scholarships for Teachers in Boston
Scholarship of $100 supported by a friend of the Laboratory since 1898.
The Edwin S. Linton Memorial Scholarship of Washington and Jefferson College.
The Bio Club Scholarship of the College of the City of New York.

5. EVENING LECTURES, 1929

Friday, June 21
Dr. Selig Hecht .... "The Nature of Visual Acuity."
Friday, June 28
Dr. Paul S. Galtsoff .... "The Physiology of Feeding and Reproduction of the American Oyster."
Friday, July 5
Dr. E. M. East .... "Immunological Phenomena in Plants."

Friday, July 12	Dr. G. H. Parker	"The Nature of Neurofibrils."
Friday, July 19	Dr. E. Fauré-Fremiet	"La Structure des amoebocytes hyalins et leurs propriétés physiologiques."
Friday, July 26	Dr. Henri Fredericq	"Humoral Transmission of Nerve Action."
Friday, August 2	Dr. P. B. Rehberg	"Studies on Kidney Function."
Friday, August 9	Dr. Hans Winterstein	"The Metabolism of the Process of Excitation."
Friday, August 16	The William Thompson Sedgwick Memorial Lecture, delivered by Dr. Torsten Thunberg	"The Hydrogen-Activating Enzymes of the Cells."
Friday, August 23	Dr. K. T. Belar	"Investigations on the Structure and Function of the Mitotic Spindle."
Friday, August 30	Dr. H. H. Dale	"Some Chemical Factors in the Regulation of the Blood Flow."
Tuesday, September 3	Dr. August Krogh	"Respiratory Mechanisms in Insects."
Friday, September 6	Dr. Joseph Barcroft	"Recent Observations on the Spleen."
Monday, September 9	The Reynold A. Spaeth Memorial Lecture, delivered by Dr. Rudolf Höber	"The Structure of the Plasma Membrane."
Wednesday, September 11	Dr. Frederic Verzar	"On the Mechanism of Absorption from the Intestine."
Friday, September 13	Dr. Enrico Sereni	"The Physiology of Chromatophores."

6. SHORTER SCIENTIFIC PAPERS, 1929

Tuesday, July 2	Dr. J. M. Johlin	"The Surface Tension of Solutions of Semicolloids in Relation to the Concentration."
	Dr. L. Michaelis	"The Mechanism of the Iron Catalysis in Oxidation."

Tuesday, July 9

Dr. Abby H. Turner .... "The Adjustment of the Human Circulation in Prolonged Standing."

Dr. W. E. Garrey .... "The Non-existence of Physiological Leucocytosis."

Dr. D. J. Edwards and
Dr. J. McKeen Cattell.......... "Some Physiological Effects of Hydrostatic Pressure."

Tuesday, July 16

Mr. L. G. Barth .... "The Effects of Acids and Alkalies on the Viscosity of Arbacia Egg Protoplasm."

Dr. L. V. Heilbrunn .... (a) "The Viscosity of Amoeba Protoplasm at Different Temperatures." (b) "Further Study of the Surface Precipitation Reaction."

Dr. Paul Reznikoff .... "Response of White Blood Cells to Stimulation."

Tuesday, July 23

Mr. Harry Grundfest .... "Visibility of the Spectrum to the Fish Eye."

Dr. Wm. L. Dolley, Jr. .... "Some Visual Phenomena in Eristalis tenax."

Dr. H. K. Hartline .... "The Course of Dark Adaptation of the Eye of Limulus as Manifested by its Electrical Response."

Tuesday, July 30

Mr. Reuben Blumenthal ...... "Some Effects of Cyanides in Relation to Mitosis in Arbacia Eggs."

Dr. Henry J. Fry .... "A Critique of the Usual Concepts Concerning the Mitotic Mechanism of the Echinoderm Egg."

Dr. Boris Sokoloff .... "Liquefaction of Malignant Tumors and Iron Metabolism."

Tuesday, August 6

Dr. A. Franklin Shull .... "Intermediate Aphids and Goldschmidt's Theory of Intersexes."

Dr. C. W. Metz .... "Unisexual Progenies and the Mechanism of Sex-determination in Sciara."

Dr. T. Dobzhansky .... "Chromosome Translocation in Drosophila Induced by X-rays."

Tuesday, August 13

Miss Helen B. Smith .... "Depression in Rotifera."

Mr. Oscar W. Richards .... "The Later Part of the Growth of a Population of Yeast."

Dr. Maynard M. Metcalf ...... "An African Zoögeographical Puzzle."

Tuesday, August 20	
Dr. Mary S. MacDougall	"Modifications in Chilodon unicinatus Produced by Ultra-violet Radiation."
Miss Rachael Bowling	"Conjugation of Distomatous Individuals of Glaucoma Scintillans."
Dr. Gary N. Calkins	"Chromosomes in Uroleptus Halseyi."
Dr. J. A. Dawson	"Motion Pictures of Living Protozoa."
Tuesday, August 27	
Dr. J. Nitzulescu, Mr. T. Kerr and Mr. G. A. Millikan	"Salt Errors of Clark-Lubs Indicators for Woods Hole Sea Water."
Dr. Sergius Morgulis	"Blood Calcium in Tetany."
Dr. S. E. Pond	'Elementary Study of the Bone Solids with Particular Reference to Age."
Dr. E. S. Guzman Barron and Dr. L. B. Flexner	"The Reduction Potential of Sugars."
Dr. Kenneth Blanchard	(a) "Inorganic Constituents of the Arbacia Egg." (b) "Autoxidation of Unsaturated Hydrocarbons."

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Coolidge, Mr. C. A., Ames Building, Boston, Mass.
Crane, Mr. C. R., New York City.
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Jackson, Mr. Chas. C., 24 Congress St., Boston, Mass.
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Lowell, Mr. A. Lawrence, 17 Quincy St., Cambridge, Mass.
Means, Dr. James Howard, 15 Chestnut St., Boston, Mass.

Merriman, Mrs. Daniel, 73 Bay State Road, Boston, Mass.
Minns, Miss Susan, 14 Louisburg Square, Boston, Mass.
Morgan, Mr. J. Pierpont, Jr., Wall and Broad Sts., New York City, N. Y.
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Morgan, Mrs. T. H., Pasadena, Calif.
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Osborn, Prof. Henry F., American Museum of Natural History, New York, N. Y.
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Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pa.
Sears, Dr. Henry F., 86 Beacon St., Boston, Mass.
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Trelease, Prof. William, University of Illinois, Urbana, Ill.
Ware, Miss Mary L., 41 Brimmer St., Boston, Mass.
Williams, Mrs. Anna P., 505 Beacon St., Boston, Mass.
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Carver, Prof. Gail L., 613 Orange St., Macon, Ga.

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Cattell, Dr. McKeen, Cornell University Medical College, New York City, N. Y.

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Colley, Mrs. Mary W., 36 Argyle Place, Rockville Centre, Long Island, N. Y.
Colton, Prof. H. S., Box 127, Flagstaff, Ariz.
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Curtis, Prof. W. C., University of Missouri, Columbia, Mo.
Davis, Dr. Donald W., College of William and Mary, Williamsburg, Va.
Davis, Dr. Alice R., 989 Memorial Drive, Cambridge, Mass.
Dawson, Dr. J. A., The College of the City of New York, New York City, N. Y.
Dederer, Dr. Pauline H., Connecticut College, New London, Conn.
Dellinger, Dr. S. C., University of Arkansas, Fayetteville, Ark.
Dodds, Prof. G. S., Medical School, University of West Virginia, Morgantown, W. Va.
Dolley, Prof. William L., University of Buffalo, Buffalo, N. Y.
Donaldson, Prof. H. H., Wistar Institute of Anatomy and Biology, Philadelphia, Pa.
Donaldson, Dr. John C., University of Pittsburgh, School of Medicine, Pittsburgh, Pa.
Drew, Prof. Gilman A., Eagle Lake, Florida.

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Gates, Dr. F. L., 31 Fayerweather St., Cambridge, Mass.
Gates, Prof. R. Ruggles, University of London, London, England.
Geiser, Dr. S. W., Southern Methodist University, Dallas, Tex.
Glaser, Prof. O. C., Amherst College, Amherst, Mass.
Glaser, Prof. R. W., Rockefeller Institute for Medical Research, Princeton, N. J.
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Harper, Prof. R. A., Columbia University, New York City, N. Y.

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Harvey, Prof. E. N., Princeton University, Princeton, N. J.

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Heath, Prof. Harold, Pacific Grove, California.

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Heilbrunn, Dr. L. V., Washington Square College, New York University.

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Hinrichs, Dr. Marie A., University of Chicago, Chicago, Ill.

Hisaw, Dr. F. L., University of Wisconsin, Madison, Wis.

Hoadley, Dr. Leigh, Harvard University, Cambridge, Mass.

Hogue, Dr. Mary J., 503 N. High St., West Chester, Pa.

Holmes, Prof. S. J., University of California, Berkeley, Calif.

Hooker, Prof. Davenport, University of Pittsburgh, Pittsburgh, Pa.

Hopkins, Dr. Hoyt S., New York University, College of Dentistry, New York City, N. Y.

Howard, Dr. Harvey J., Washington University, St. Louis, Mo.

Howe, Dr. H. E., 2702 36th St., N. W., Washington, D. C.

Hoyt, Dr. William D., Washington and Lee University, Lexington, Va.

Humphrey, Mr. R. R., University of Buffalo, School of Medicine, Buffalo, N. Y.

Hyman, Dr. Libbie H., University of Chicago, Chicago, Ill.

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Irwin, Dr. Marian, Rockefeller Institute, New York City, N. Y.

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Jacobs, Prof. Merkel H., University of Pennsylvania Medical School, Philadelphia, Pa.

Jennings, Prof. H. S., Johns Hopkins University, Baltimore, Md.

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Johnson, Prof. George E., State Agricultural College, Manhattan, Kans.

Jones, Prof. Lynds, Oberlin College, Oberlin, O.

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Kennedy, Dr. Harris, Readville, Mass.
Kindred, Dr. J. E., University of Virginia, Charlottesville, Va.
King, Dr. Helen D., Wistar Institute of Anatomy and Biology, Philadelphia, Pa.
King, Dr. Robert L., University of Pennsylvania, Philadelphia, Pa.
Kingsbury, Prof. B. F., Cornell University, Ithaca, N. Y.
Kirkham, Dr. W. B., Springfield College, Springfield, Mass.
Knapke, Rev. Bede, St. Bernard's College, St. Bernard, Ala.
Knower, Prof. H. McE., Wistar Institute of Anatomy and Biology, Philadelphia, Pa.
Knowlton, Prof. F. P., Syracuse University, Syracuse, N. Y.
Kostir, Dr. W. J., Ohio State University, Columbus, O.
Kribs, Dr. Herbert, 202A Copley Road, Upper Darby, Pa.
Kuyk, Dr. Margaret P., Westbrook Ave., Richmond, Va.
Lancefield, Dr. D. E., Columbia University, New York City, N. Y.
Lange, Dr. Mathilde M., Wheaton College, Norton, Mass.
Lee, Prof. F. S., College of Physicians and Surgeons, New York City, N. Y.
Lewis, Prof. I. F., University of Virginia, Charlottesville, Va.
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Md.
Lillie, Prof. Frank R., University of Chicago, Chicago, Ill.
Lillie, Prof. Ralph S., University of Chicago, Chicago, Ill.
Linton, Prof. Edwin, University of Pennsylvania, Philadelphia, Pa.
Loeb, Prof. Leo, Washington University Medical School, St. Louis, Mo.
Loeb, Mrs. Leo, 812 Boland Place, St. Louis, Mo.
Lowther, Mrs. Florence DeL., Barnard College, Columbia University, New York City, N. Y.
Lucké, Prof. Balduin, University of Pennsylvania, Philadelphia. Pa.
Lund, Dr. E. J., University of Texas, Austin, Tex.
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Lynch, Dr. Ruth Stocking, Johns Hopkins University, Baltimore, Md.
Lyon, Prof. E. P., University of Minnesota, Minneapolis, Minn.
MacCallum, Dr. G. A., 925 St. Paul St., Baltimore, Md.
MacDougall, Dr. Mary S., Agnes Scott College, Decatur, Ga.
McClung, Prof. C. E., University of Pennsylvania, Philadelphia, Pa.
McGee, Dr. Anita Newcomb, Box 363, Southern Pines, N. C.
McGregor, Dr. J. H., Columbia University, New York City, N. Y.
McMurrich, Prof. J. P., University of Toronto, Toronto, Canada.

McNair, Dr. G. T., 1624 Alabama St., Lawrence, Kans.

Macklin, Dr. Charles C., School of Medicine, University of Western Ontario, London, Canada.

Malone, Prof. E. F., University of Cincinnati, Cincinnati, O.

Manwell, Dr. Reginald D., School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Md.

Martin, Prof. E. A., College of the City of New York, New York City, N. Y.

Mast, Prof. S. O., Johns Hopkins University, Baltimore, Md.

Mathews, Prof. A. P., University of Cincinnati, Cincinnati, O.

Matsui, Prof. K., Imperial College of Agriculture and Dendrology, Morioka, Japan.

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Meigs, Dr. E. B., Dairy Division Experiment Station, Beltsville, Md.

Meigs, Mrs. E. B., 1736 M St., N. W., Washington, D. C.

Metcalf, Prof. M. M., Johns Hopkins University, Baltimore, Md.

Metz, Prof. Charles W., Carnegie Institution of Washington, Cold Spring Harbor, Long Island, N. Y.

Michaelis, Dr. Leonor, Johns Hopkins University, Baltimore, Md.

Miner, Dr. Roy W., American Museum of Natural History, New York City, N. Y.

Mitchell, Dr. Philip H., Brown University, Providence, R. I.

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Moore, Prof. George T., Missouri Botanical Garden, St. Louis, Mo.

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Morrill, Prof. C. V., Cornell University Medical College, New York City, N. Y.

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Nabours, Dr. R. K., Kansas State Agricultural College, Manhattan, Kans.

Nachtrieb, Prof. Henry F., 2448 Cedar St., Berkeley, Calif.

Neal, Prof. H. V., Tufts College, Tufts College, Mass.

Newman, Prof. H. H., University of Chicago, Chicago, Ill.

Nichols, Dr. M. Louise, Powelton Apartments, Philadelphia, Pa.

Noble, Dr. Gladwyn K., American Museum of Natural History, New York City, N. Y.

Nonidez, Dr. Jose F., Cornell University Medical College, New York City, N. Y.

Okkelberg, Dr. Peter, University of Michigan, Ann Arbor, Mich.
Osburn, Prof. R. C., Ohio State University, Columbus, O.
Osterhaut, Prof. W. J. V., Rockefeller Institute, New York City, N. Y.
Packard, Dr. Charles, Columbia University, Institute of Cancer Research, 1145 Amsterdam Ave., New York City, N. Y.
Page, Dr. Irvine H., Presbyterian Hospital, New York City, N. Y.
Papanicolaou, Dr. George N., Cornell University Medical College, New York City, N. Y.
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Patten, Prof. William, Dartmouth College, Hanover, N. H.
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Payne, Prof. F., University of Indiana, Bloomington, Ind.
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Pearse, Prof. A. S., Duke University, Durham, N. C.
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Phillips, Dr. Ruth L., Western College, Oxford, O.
Pike, Prof. Frank H., 437 West 59th St., New York City, N. Y.
Pinney, Dr. Mary E., Milwaukee-Downer College, Milwaukee, Wis.
Plough, Prof. Harold H., Amherst College, Amherst, Mass.
Pond, Dr. Samuel E., University of Pennsylvania, School of Medicine, Philadelphia, Pa.
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Rand, Dr. Herbert W., Harvard University, Cambridge, Mass.
Rankin, Prof. W. M., Princeton University, Princeton, N. J.
Redfield, Dr. Alfred C., Harvard University Medical School, Boston, Mass.
Reese, Prof. Albert M., West Virginia University, Morgantown, W. Va.
Reinke, Dr. E. E., Vanderbilt University, Nashville, Tenn.
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Richards, Prof. A., University of Oklahoma, Norman, Oklahoma.

Riggs, Mr. Lawrason, Jr., 25 Broad St., New York City, N. Y.
Robertson, Prof. W. R. B., Columbia, Mo.
Rogers, Prof. Charles G., Oberlin College, Oberlin, O.
Romer, Dr. Alfred S., University of Chicago, Chicago, Ill.
Sampson, Dr. Myra M., Smith College, Northampton, Mass.
Sands, Miss Adelaide G., 562 King St., Port Chester, N. Y.
Schrader, Dr. Franz, Bryn Mawr College, Bryn Mawr, Pa.
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Scott, Dr. Ernest L., Columbia University, New York City, N. Y.
Scott, Prof. G. G., College of the City of New York, New York City, N. Y.
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Scott, Prof. William B., 7 Cleveland Lane, Princeton, N. J.
Shull, Prof. A. Franklin, University of Michigan, Ann Arbor, Mich.
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Snyder, Prof. Charles D., Johns Hopkins University Medical School, Baltimore, Md.
Sollman, Dr. Torald, Western Reserve University, Cleveland, O.
Speidel, Dr. Carl C., University of Virginia, Charlottesville, Va.
Spencer, Prof. H. J., 24 West 10th St., New York City, N. Y.
Stark, Dr. Mary B., New York Homeopathic Medical College and Flower Hospital, New York City, N. Y.
Stockard, Prof. C. R., Cornell University Medical College, New York City, N. Y.
Stokey, Dr. Alma G., Mount Holyoke College, South Hadley, Mass.
Streeter, Prof. George L., Carnegie Institution, Baltimore, Md.
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Stunkard, Dr. Horace W., New York University, University Heights, N. Y.
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Tashiro, Dr. Shiro, Medical College, University of Cincinnati, Cincinnati, O.
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Taylor, Dr. William R., University of Pennsylvania, Philadelphia, Pa.
Tennent, Prof. D. H., Bryn Mawr College, Bryn Mawr, Pa.
Thatcher, Mr. Lloyd E., University of Mississippi, University, Miss.
Tinkham, Miss Florence L., 71 Ingersoll Grove, Springfield, Mass.
Tracey, Prof. Henry C., University of Kansas, Lawrence, Kans.

TREADWELL, PROF. A. L., Vassar College Poughkeepsie, N. Y.
TURNER, PROF. C. L., Northwestern University, Evanstown, Ill.
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UHLENHUTH, DR. EDUARD, University of Maryland, School of Medicine, Baltimore, Md.
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VAN DER HEYDE, DR. H. C., Galeria, Corse, France.
VISSCHER, DR. J. PAUL, Western Reserve University, Cleveland, O.
WAITE, PROF. F. C., Western Reserve University Medical School, Cleveland, O.
WALLACE, DR. LOUISE B., Spelman College, Atlanta, Ga.
WARD, PROF. HENRY B., University of Illinois, Urbana, Ill.
WARDWELL, DR. E. H., Chappaqua, N. Y.
WARREN, PROF. HOWARD C., Princeton University, Princeton, N. J.
WARREN, DR. HERBERT S., University of Idaho, Moscow, Ida.
WENRICH, DR. H., University of Pennsylvania, Philadelphia, Pa.
WHEDON, DR. A. D., North Dakota Agricultural College, Fargo, N. D.
WHEELER, PROF. W. M., Bussey Institution, Forest Hills, Mass.
WHERRY, DR. W. B., Cincinnati Hospital, Cincinnati, O.
WHITAKER, DR. DOUGLAS M., Columbia University, New York City, N. Y.
WHITE, DR. E. GRACE, Wilson College, Chambersburg, Pa.
WHITESIDE, DR. BEATRICE, Detroit College of Medicine and Surgery, Detroit, Mich.
WHITING, DR. PHINEAS W., University of Pittsburgh, Pittsburgh, Pa.
WHITNEY, DR. DAVID D., University of Nebraska, Lincoln, Nebr.
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WILLIER, DR. B. H., University of Chicago, Chicago, Ill.
WILSON, PROF. H. V., University of North Carolina, Chapel Hill, N. C.
WILSON, DR. J. W., Brown University, Providence, R. I.
WOGLOM, PROF. WILLIAM H., Columbia University, New York City, N. Y.
WOODRUFF, PROF. L. L., Yale University, New Haven, Conn.
WOODWARD, DR. ALVALYN E., Zoölogy Department, Ann Arbor, Mich.
YOUNG, DR. B. P., Cornell University, Ithaca, N. Y.
YOUNG, DR. D. B., University of Maine, Orono, Me.
ZELENY, DR. CHARLES, University of Illinois, Urbana, Ill.

THE INFLUENCE OF CARBON DIOXIDE UPON THE OXYGEN CONSUMPTION OF PARAMECIUM AND THE EGG OF ARBACIA

WALTER S. ROOT

(From the Department of Physiology, School of Medicine, University of Pennsylvania, the Marine Biological Laboratory, Woods Hole, Mass., and the Department of Physiology, School of Medicine, Syracuse University)

The inter-relationship between oxygen and carbon dioxide in supporting the respiration of higher animals is a matter of fundamental importance. Since experiments upon such forms are often difficult to interpret, because of the number of uncontrollable factors involved, single cells have been used in the present study in which the material employed was *Paramecium caudatum* and the fertilized eggs of *Arbacia punctulata*.

Few investigations have been made concerning the effect of carbon dioxide upon the oxygen consumption of protozoa or of marine eggs, though Warburg (1910) observed no change in the respiration of fertilized sea urchin eggs in the presence of a carbon dioxide tension of 15 mm. Hg, and Burfield (1928) reported that small amounts of carbon dioxide profoundly decreased the rate of oxygen consumption of plaice eggs. In these studies, however, oxygen was measured by the Winkler method which, while accurate for the determination of dissolved oxygen in solutions free of organic materials, is said to be untrustworthy for egg suspensions (Heilbrunn, 1915; Warburg, 1914*a*). Moreover, the presence of iron in sea urchin eggs (Warburg, 1914*b*) is known to introduce large errors into the method (Alsterberg, 1926). Previous work on the effect of carbon dioxide upon the rate of oxygen consumption of single cells appears, therefore, to be not entirely satisfactory and a reinvestigation of the question by other methods has seemed desirable. In the experiments here recorded, the technique employed was that developed by Novy and his collaborators (1925) for bacterial respiration, and modified by Amberson (1928) for the respiration of unicellular animal organisms.

Experiments with Paramecium

A thick suspension of the protozoa was obtained by placing several liters of culture in a large glass cylinder which was illuminated near the top by rays of light from a sixty-watt electric light bulb. The organisms under these conditions swim to the top, and may be siphoned off in great numbers. The suspension so obtained was then centrifuged at two thousand revolutions a minute for ten to twenty seconds. The centrifuge was stopped suddenly to prevent the organisms from swimming from the bottom of the centrifuge tubes before the supernatant fluid could be decanted. The sediment of protozoa was washed in several changes of boiled, cooled, filtered pond water. The suspension in its final form must have been relatively free from bacteria, because control experiments with the cells absent showed no measurable oxygen consumption. The cultures were never pure, but *P. caudatum* always constituted at least 95 per cent of the protozoa present. The original culture of *Paramecium* was obtained from Dr. William Canovan of the Zoölogy Department of the University of Pennsylvania.

In order to obtain two suspensions containing approximately the same number of cells, a calibrated glass " mixer " was used. This consisted of a glass tube three-quarters of an inch in diameter, fitted at each end with a ground glass stopper. It was separated into two chambers of about twenty cc. capacity by a stopcock, the bore of which was of the same diameter as the tube. The suspension was poured into and out of the " mixer " eight to ten times with one stopper in place and with the stopcock open. After pouring the suspension in for the last time, the second stopper was inserted and the stopcock turned before the protozoa had an opportunity to change their distribution. The suspensions on each side of the stopcock were then poured into two calibrated cylindrical glass vessels of about the size and shape of Haldane gas collecting tubes. These tonometers were fitted with three-way stopcocks at both ends. Twenty-five cc. of boiled, cooled, filtered pond water were added to each tonometer.

Gas mixtures were made up in two twenty-one liter bottles so arranged that, when air or nitrogen was forced into one, the water contained therein passed into the second bottle, displacing the gas mixture which had previously been made up to approximately the desired percentage of oxygen and carbon dioxide. While the water contained in these bottles absorbed a certain amount of the gas mixtures above it, the gas tensions were determined from samples of the gas after it had passed through the tonometer, and immediately before the tonometer was closed. Any changes in gas tension occurring within the bottles did not, therefore, result in errors in the respiratory determinations.

At the beginning of an experiment, the gas mixture was slowly bubbled through one of the suspensions. Temperature equilibrium was achieved by placing the tonometers in a water bath at the same temperature as that at which the respiration was to be measured (25.6° ± 0.3° C.). Every few seconds the passage of the gas was suspended and, with the stopcocks closed, the tonometer was gently rocked to hasten the attainment of equilibrium. After such a procedure, a drop of water was left in the capillary part of the tonometer which leads to the stopcock. When the tonometer was opened to the outside air, the drop was displaced inwards, because the pressure within the tonometer was less than atmospheric, due to the absorption of gases by the suspension. Passage of the gas mixture was continued until the drop was no longer displaced. A sample of the gas mixture, after it had passed through the tonometer, was now collected in a Bailey sampling bottle. Care was exercised to prevent the presence of a positive pressure within the tonometer. The stopcocks were then closed and the tonometers were placed in a second water bath. The tonometers were rotated upon their long axes sixty times a minute. No cellular destruction or abnormal behavior was observed. The same cultures were used every second or third day.

While the first suspension was undergoing the above treatment, the second suspension was equilibrated in the same water bath with air, by connecting one end of the tonometer with a compressed air inlet or a water pump. After approximately the same length of time as was required for the equilibration of the tonometer containing carbon dioxide, an air sample was secured, the stopcocks were closed, and the tonometer was placed in the rotator. The two tonometers rarely differed by more than five to ten minutes in their starting times. At the end of two to four hours, the tonometers were removed from the water bath and a sample of the contained gas was withdrawn in a Bailey collector, and set aside for later analysis. Respiratory exchanges were calculated for three-hour experiments.

The gas samples were analyzed in duplicate by means of a Haldane-Henderson analyzer with a nitrogen side tube, the principle being that employed in Bazett's modification (1928). The analyses were controlled by daily air analyses, and were accurate to 0.03 to 0.04 per cent.

Assuming the gaseous solubilities to be those given by the standard tables for pure water at this temperature, the total oxygen and carbon dioxide present at the beginning and at the end of the experiment in both air and water were calculated, the usual correction for barometer, water vapor, etc., being applied. The results of a typical experiment are as follows:

Sample Experiment

Tonometers	VI	V
Volume of tonometers	99.36 cc.	99.47 cc.
Volume of suspension	45.01 cc.	45.01 cc.
Gas-analysis at beginning		
O_2	20.91%	19.29%
CO_2	0.04%	7.50%
N_2	79.05%	73.21%
Gas analysis at end (corrected for volume change)		
O_2	18.71%	17.22%
CO_2	0.88%	8.61%
N_2	79.05%	73.21%
Oxygen in air and water		
at beginning	11.626 cc.	10.746 cc.
at end	10.403 cc.	9.593 cc.
Oxygen consumption	1.223 cc.	1.153 cc.
Carbon dioxide in air and water		
at beginning	0.036 cc.	6.788 cc.
at end	0.793 cc.	7.789 cc.
Carbon dioxide production	0.757 cc.	1.001 cc.
Volumes corrected to dry values at 0° C. and 760 mm. Hg		
Oxygen consumption	1.081 cc.	1.019 cc.
Carbon dioxide production	0.669 cc.	0.885 cc.
Respiratory quotient	0.618	0.868
Oxygen tension in mm. Hg	153.4–137.2	141.5–126.3
Carbon dioxide tension in mm. Hg	0.3– 6.4	55.0–63.1

Ratio oxygen consumption $\frac{V}{VI} = 0.942$

Ratio carbon dioxide production $\frac{V}{VI} = 1.322$

The results obtained in forty-five experiments are given in Table I and Fig. 1. It will be noted that at a carbon dioxide tension of about 15 mm. Hg, the curve of the rate of oxygen consumption rises, reaching a maximum at approximately 40 mm. Hg and, crossing the line of the control rate of oxygen consumption at about 67 mm. Hg, falls away at the higher tensions.

Examination of the tonometers with a low powered binocular microscope showed that an exposure of three hours to a carbon dioxide tension of about 150 mm. Hg noticeably decreased the motility of the protozoa. At about 220 mm. Hg the cells became shorter and thicker and the nuclei became more clearly visible, standing out sharply from the rest of the protoplasm. When the animals were subjected to a tension of 250 mm. Hg for three hours, some were irreversibly affected by the gas.

In all experiments, the oxygen tension was kept above 62 mm. Hg. Amberson (1928) has shown that the rate of oxygen consumption of *Paramecium* is constant between 200 and 50 mm. Hg partial pressure, so it is unlikely that the results here reported were due to a lack of

TABLE I

Respiration of Paramecium at Different Carbon Dioxide Tensions

Experiment No.	CO$_2$ Tensions in Tonometer B	Respiration in Tonometer A			Respiration in Tonometer B			Ratio O$_2$ Consumption $\frac{B}{A}$	Ratio CO$_2$ Production $\frac{B}{A}$
		O$_2$ Consumed	CO$_2$ Produced	*RQ*	O$_2$ Consumed	CO$_2$ Produced	*RQ*		
	mm. Hg	*cc.*	*cc.*		*cc.*	*cc.*			
1	0.4– 3.6	0.381	0.256	0.669	0.357	0.254	0.711	0.936	0.999
2	0.3– 12.1	1.159	0.871	0.751	1.116	0.924	0.827	0.962	1.060
3	0.4– 7.4	1.169	0.731	0.625	1.154	0.692	0.599	0.987	0.946
4	1.0– 8.1	1.264	0.647	0.511	1.302	0.732	0.562	0.970	1.131
5	16.7– 26.4	1.305	0.848	0.650	1.315	1.042	0.780	1.007	1.228
6	25.8– 33.9	0.776	0.535	0.691	0.817	0.895	1.096	1.052	1.672
7	29.1– 39.1	1.232	0.813	0.659	1.359	1.083	0.796	1.103	1.332
8	34.4– 37.4	0.410	0.288	0.702	0.452	0.347	0.729	1.102	1.204
9	39.3– 43.1	0.592	0.266	0.450	0.652	0.315	0.484	1.101	1.184
10	42.7– 50.3	0.813	0.474	0.613	0.907	0.713	0.823	1.115	1.504
11	43.0– 52.3	1.565	0.810	0.525	1.628	1.004	0.617	1.040	1.239
12	44.8– 54.2	0.813	0.511	0.617	0.886	1.012	1.144	1.067	1.980
13	48.0– 49.8	0.572	0.296	0.519	0.574	0.181	0.318	1.004	0.611
14	50.9– 58.2	1.314	0.789	0.601	1.485	0.779	0.525	1.130	0.987
15	52.4– 61.4	1.235	0.776	0.619	1.312	0.977	0.740	1.047	1.259
16	62.5– 69.5	1.117	0.599	0.536	1.187	0.763	0.642	1.062	1.273
17	69.3– 75.3	1.622	0.923	0.569	1.504	0.651	0.432	0.927	0.705
18	69.8– 79.0	1.674	0.925	0.552	1.573	0.889	0.565	0.939	0.961
19	80.5– 90.9	2.370	1.119	0.472	2.118	1.142	0.539	0.893	1.020
20	90.6– 97.3	0.774	0.554	0.716	0.644	0.662	1.027	0.832	1.176
21	91.4–110.8	3.260	1.859	0.570	3.021	2.018	0.668	0.926	1.085
22	94.7–100.9	0.864	0.643	0.744	0.810	0.589	0.727	0.937	0.916
23	102.6–118.8	2.417	1.448	0.599	2.222	1.689	0.760	0.919	1.166
24	105.6–127.4	3.839	2.152	0.565	3.495	2.334	0.667	0.910	1.089
25	126.7–135.3	0.863	0.565	0.654	0.736	0.814	1.105	0.852	1.440
26	140.9–149.9	1.197	0.812	0.678	0.950	0.964	1.014	0.793	1.187
27	141.6–151.4	0.872	0.645	0.739	0.594	1.071	1.803	0.681	1.660
28	155.0–164.8	1.228	0.789	0.642	0.927	1.004	1.083	0.754	1.272
29	158.2–166.2	1.710	1.015	0.593	1.333	1.213	0.909	0.779	1.195
30	160.8–171.2	1.246	0.888	0.712	0.791	1.027	1.298	0.634	1.156
31	167.3–173.7	0.827	0.514	0.621	0.534	0.473	0.885	0.645	0.920
32	173.8–185.3	2.196	1.308	0.595	1.494	1.334	0.893	0.680	1.019
33	181.8–192.8	1.293	0.997	0.771	0.892	0.867	0.972	0.689	0.869
34	202.2–204.1	0.970	0.667	0.687	0.506	0.184	0.363	0.521	0.275
35	206.8–210.8	1.499	0.946	0.631	0.702	0.395	0.562	0.469	0.418
36	217.4–222.0	2.032	1.124	0.553	1.004	0.525	0.521	0.494	0.467
37	224.7–229.4	0.828	0.488	0.589	0.511	0.502	0.982	0.617	1.028
38	224.9–233.5	0.918	0.509	0.554	0.317	0.914	2.812	0.345	1.795
39	234.5–241.2	0.610	0.385	0.631	0.360	0.713	1.980	0.590	1.851
40	238.2–246.8	0.990	0.673	0.679	0.504	0.713	1.414	0.509	1.059
41	253.3–257.1	1.296	0.679	0.524	0.556	0.385	0.692	0.429	0.567
42	270.2–276.9	1.177	0.787	0.629	0.454	0.693	1.526	0.385	0.880
43	277.2–296.7	1.606	1.010	0.628	0.852	1.711	2.008	0.530	1.694
44	353.9–361.0	1.558	1.017	0.652	0.843	0.715	0.848	0.541	0.703
45	418.1–423.8	0.860	0.398	0.462	0.288	0.599	2.079	0.334	1.505

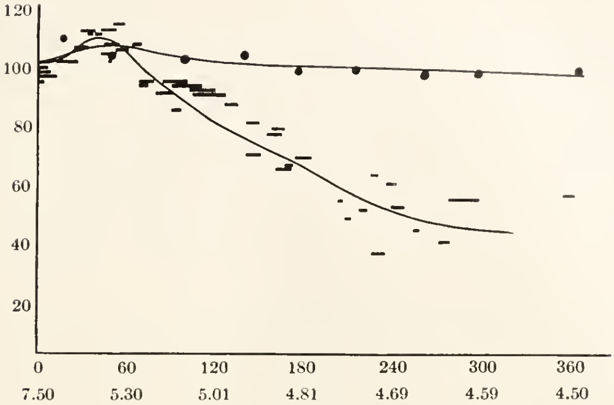

Fig. 1. The effect of carbon dioxide upon the oxygen consumption of *Paramecium*.

Abscissa: upper row of figures = mm. Hg carbon dioxide tension,
lower row of figures = pH.

$$\text{Ordinate} = \frac{\text{oxygen consumption in carbon dioxide tonometer}}{\text{oxygen consumption in control or air tonometer}}.$$

— = carbon dioxide experiments.
● = hydrochloric acid experiments.

oxygen. It is, of course, possible that the oxygen tension at which the effects of oxygen deficiency appear, may not be the same in the presence of high tensions of carbon dioxide as in the relative absence of this gas. If the above experiments had been carried out in the region of oxygen deficiency, however, even slight increases in the oxygen tension would have resulted in an increased rate of oxygen consumption. That this was not the case is shown by a comparison of experiments 18 and 19 (Table II) which show that when the oxygen tension in tonometer *B* (CO_2) in experiment 18 was 62 per cent greater than that in tonometer *B* (CO_2), experiment 19, the differences in the rates of oxygen consumption lay within the error of the method. The carbon dioxide tensions in the two tonometers B (CO_2) do not differ by enough to affect appreciably the rates of the oxygen consumption in the two experiments.

Table II

Experiment No.	CO_2 tension	Tonometer A O_2 tension	O_2 Consumed	CO_2 tension	Tonometer B O_2 tension	O_2 Consumed	Ratio O_2 Consumption B/A
	mm. Hg	*mm. Hg*		*mm. Hg*	*mm. Hg*		
18	0.30– 9.20	156.5–130.3	1.758	69.8–79.0	142.27–115.8	1.504	0.939
19	0.30–10.64	156.8–121.4	2.370	80.5–90.9	93.8 – 62.4	1.774	0.893

The average of the respiratory quotients obtained in air in forty-eight determinations was 0.62. This is somewhat lower than the average value 0.69 obtained by Amberson (1928) in fourteen experiments. An examination of the respiratory quotients in the presence of different carbon dioxide tensions (Table I) shows that these values fluctuate irregularly between 0.32 and 2.81. When the forty-two experiments, carried out at different carbon dioxide tensions, and their controls are arranged in seven groups of six experiments each, and the respiratory quotients, and ratios of oxygen consumption and carbon dioxide production averaged, Table III is obtained. It will be seen that the rate of carbon dioxide production in the presence of carbon dioxide does not decrease as does the rate of oxygen consumption, but remains relatively constant. As a consequence, the respiratory quotients rise progressively as the carbon dioxide tension is increased.

Table III

Summary of Experiments in Table I (Paramecium caudatum)

Group	Experiments	CO_2 tension mm. Hg	*RQ* control	*RQ* CO_2 tonometer	Ratio O_2 Consumption $\frac{CO_2 \text{ tonometer}}{\text{control}}$	Ratio CO_2 Production $\frac{CO_2 \text{ tonometer}}{\text{control}}$
I.....	4–9	1.0– 43.1	0.610	0.741	1.056	1.289
II....	10–15	42.7– 61.4	0.582	0.694	1.067	1.096
III...	16–21	62.0–110.8	0.569	0.645	0.929	1.036
IV...	22–27	91.4–151.4	0.663	1.012	0.848	1.242
V....	28–33	155.0–192.0	0.655	1.006	0.696	1.072
VI...	34–39	202.0–246.0	0.574	1.205	0.506	0.972
VII..	40–45	238.0–423.0	0.595	1.428	0.454	1.068

It has been clearly shown that carbon dioxide enters cells with ease (Jacobs 1920*a*, 1920*b*, 1924), whereas hydrochloric acid penetrates cells very slowly, if at all (Loeb, 1909, Jacobs 1924). In order to determine whether carbon dioxide decreases the rate of oxygen consumption by some internal action on the cell, or merely by increasing the acidity of the external solution, a group of nine experiments was performed to determine the effects upon the respiration of *Paramecium* of approximately the same degrees of acidity produced by hydrochloric acid.

Boiled, filtered, cooled, pond water was equilibrated at different carbon dioxide tensions, and the hydrogen ion concentrations determined electrometrically, using a closed quinhydrone electrode (Fig. 2). The pond water, though poorly buffered, contained bicarbonate in a concentration approximately 0.00014 M. This was sufficient to give relatively stable potentials. The pH readings were accurate to 0.04 of a pH unit as shown by determinations made upon known solutions and by comparing a series of readings.

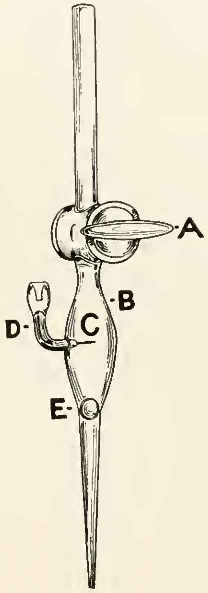

Fig. 2. Closed quinhydrone electrode.

To obtain hydrogen ion concentrations by means of hydrochloric acid, corresponding to those produced by carbon dioxide, pond water was made bicarbonate free by adding concentrated hydrochloric acid to bring it to a pH of 3 to 4, and aerated over night. It was then returned to the desired pH by the addition of concentrated sodium hydroxide.

The calculations of oxygen consumption and carbon dioxide production at different pH values appear in Table IV and Fig. 1. These results indicate that hydrochloric acid has no effect upon the respiration of *P. caudatum* at pH values as low as 4.5. The protozoa appeared in no way injured by these experiments.

TABLE IV

The Effect of Hydrochloric Acid on the Respiration of Paramecium

Experiment No.	Tonometer B pH	Resp. in Tonometer A			Resp. in Tonometer B			Ratio O_2 Consumption $\frac{B}{A}$
		O_2 Consumed	CO_2 Produced	*RQ*	O_2 Consumed	CO_2 Produced	*RQ*	
		cc.	*cc.*		*cc.*	*cc.*		
1	5.85	0.752	0.626	0.832	0.813	0.606	0.745	1.081
2	5.40	0.863	0.526	0.609	0.887	0.549	0.618	1.026
3	5.07	1.113	0.673	0.604	1.119	0.628	0.561	1.005
4	4.90	0.970	0.473	0.487	0.992	0.513	0.517	1.022
5	4.80	1.202	0.830	0.690	1.157	0.875	0.756	0.962
6	4.71	0.805	0.614	0.762	0.783	0.617	0.786	0.972
7	4.61	1.229	0.868	0.706	1.178	0.928	0.787	0.958
8	4.55	0.977	0.561	0.574	0.942	0.659	0.699	0.964
9	4.50	0.618	0.466	0.754	0.610	0.540	0.885	0.987

EXPERIMENTS ON FERTILIZED ARBACIA EGGS

Since it was impossible to determine how much of the depression in the rate of oxygen consumption of *Paramecium* was due to a suppression of the oxidative mechanism, and how much was dependent upon the decreased motility of the organism, it was necessary to perform similar experiments upon a non-motile cell. Experiments were, therefore, carried out on the fertilized eggs of *Arbacia punctulata* at Woods Hole.

The eggs were removed from the female, freed of ovarian debris by straining through cheese cloth, and washed in several changes of sea water. A heavy suspension of cells was secured by allowing the eggs to sediment in several finger bowls, and decanting the supernatant sea water. Eggs from twelve to fifteen females were used in each experiment. After fertilization, the excess spermatozoa were removed by allowing the cells to sediment in several changes of sea water. The suspension was then divided into two parts by means of the " mixer " used in the experiments with *Paramecium*. The two suspensions were equal to within 10 per cent as measured by the respiratory exchange in control experiments. The tonometers used in the experiments on *Paramecium* were also employed in these experiments.

At times varying from twenty to sixty minutes after fertilization, the suspensions were equilibrated simultaneously with air and with different carbon dioxide tensions in the same manner as described for *Paramecium*. At the conclusion of the equilibration in the water bath, the two tonometers were closed in such a manner that the contained gas was left at atmospheric pressure and at a temperature of 20.6° ±

0.3° C. Initial gas samples were secured in Bailey bottles as in the experiments with *Paramecium.* The tubes were then rotated on their long axes thirty to sixty times a minute at the equilibration temperature. Under these conditions the eggs were evenly distributed throughout the suspension. Cleavage proceeded in the normal manner, but it was somewhat delayed in time. A quantitative study of the retardation in cleavage at different carbon dioxide tensions will be published elsewhere by Haywood and Root (1930). Eighty to one hundred per cent cleavage was obtained in all the air tonometers except those indicated in Table V. Cytolysis during the experiments was not observed when fertilized eggs were used, although unfertilized eggs were found to be extremely fragile.

TABLE V

Respiration of Fertilized Arbacia Eggs at Different Carbon Dioxide Tensions

Experiment No.	CO_2 Tensions in Tonometer B	Respiration in Tonometer A			Respiration in Tonometer B			Ratio O_2 Consumption $\frac{B}{A}$	Ratio CO_2 Production $\frac{B}{A}$
		O_2 Consumed	CO_2 Produced	*RQ*	O_2 Consumed	CO_2 Produced	*RQ*		
	mm. Hg	*cc.*	*cc.*		*cc.*	*cc.*			
1	0.38– 2.92	0.722	0.346	0.479	0.664	0.318	0.486	0.920	0.919
2	0.30– 7.85	1.069	0.803	0.751	1.005	0.848	0.843	0.940	1.056
3	0.30– 5.03	0.816	0.504	0.619	0.762	0.550	0.722	0.934	1.091
4*	1.27– 10.07	1.285	0.963	0.749	1.161	0.844	0.726	0.903	0.876
5	1.55– 6.55	0.471	0.401	0.851	0.446	0.365	0.817	0.946	0.910
6	6.72– 10.82	1.031	0.734	0.711	0.823	0.436	0.529	0.798	0.594
7	6.97– 14.24	1.319	0.924	0.700	1.022	1.086	1.062	0.774	1.175
8	9.74– 19.77	2.406	1.564	0.650	1.755	1.178	0.671	0.722	0.753
9	12.58– 14.72	0.456	0.335	0.734	0.320	0.228	0.712	0.702	0.680
10*	16.23– 21.51	1.823	1.380	0.757	0.964	0.559	0.579	0.529	0.405
11	19.96– 25.19	1.794	1.163	0.648	0.993	0.603	0.607	0.553	0.518
12	25.40– 27.03	0.506	0.345	0.681	0.220	0.172	0.786	0.434	0.498
13	27.58– 29.28	1.154	0.848	0.734	0.426	0.175	0.410	0.369	0.206
14	34.67– 37.88	1.013	0.646	0.637	0.295	0.345	1.169	0.291	0.534
15	37.80– 40.68	1.103	0.910	0.804	0.324	0.306	0.928	0.293	0.336
16	42.33– 44.57	0.925	0.683	0.738	0.206	0.232	1.126	0.222	0.339
17	43.80– 45.07	0.608	0.359	0.590	0.173	0.131	0.754	0.284	0.365
18	51.73– 52.48	0.629	0.575	0.914	0.144	0.081	0.562	0.229	0.140
19	58.43– 60.16	1.111	0.761	0.685	0.205	0.181	0.882	0.184	0.236
20	63.93– 65.14	0.999	0.829	0.829	0.339	0.131	0.386	0.339	0.157
21	64.59– 65.49	0.883	0.629	0.712	0.187	0.101	0.540	0.211	0.160
22	71.51– 72.56	0.860	0.588	0.683	0.197	0.112	0.577	0.229	0.190
23*	85.50– 87.94	0.992	0.729	0.734	0.343	0.192	0.560	0.345	0.263
24*	86.90– 88.02	0.615	0.439	0.713	0.178	0.119	0.669	0.289	0.271
25	124.99–125.75	0.558	0.356	0.638	0.185	0.075	0.405	0.331	0.210
26	131.61–133.33	0.793	0.570	0.718	0.143	0.184	1.286	0.180	0.322
27	139.79–140.41	0.543	0.387	0.712	0.147	0.065	0.442	0.270	0.168
28*	162.92–163.15	0.972	0.775	0.797	0.176	0.024	0.136	0.181	0.030
29	176.02–177.19	0.558	0.413	0.740	0.045	0.125	0.277	0.081	0.302

* Less than 80 per cent cleavage in air tubes.

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In the experiments whose duration deviated from three hours, the respiratory exchange was calculated for this time. At 20° C., the first cell division occurred about one hour after fertilization in the control tonometers. At the end of two hours the cells were in the four and eight cell stages; at the end of three hours, they were in the sixteen and thirty-two cell stages. The material was not, therefore, unicellular throughout the whole experiment. No differences were observed in the effects of carbon dioxide upon the rate of oxygen consumption in experiments in which the egg had started to cleave before introduction into the tonometers, as compared with eggs which had not started to cleave at that time.

At the end of the experiment, samples of gas were secured from both tonometers and analyzed. The amounts of oxygen and of carbon dioxide in the gas and in the sea water were then calculated for the beginning and for the end of the experiments. For this calculation, the absorption coefficients for oxygen and carbon dioxide in sea water given by Krümmel (1907) were used.

The results obtained in twenty-nine experiments are given in Table V and shown graphically in Fig. 3. It will be observed in Fig. 3 that, for each 10 mm. increase in the carbon dioxide tension up to 30 mm. Hg, there is a 21 per cent decrease in the rate of oxygen consumption. Above 30 mm. Hg, further increases in the carbon dioxide tension have a relatively slight effect upon the rate of oxygen consumption.

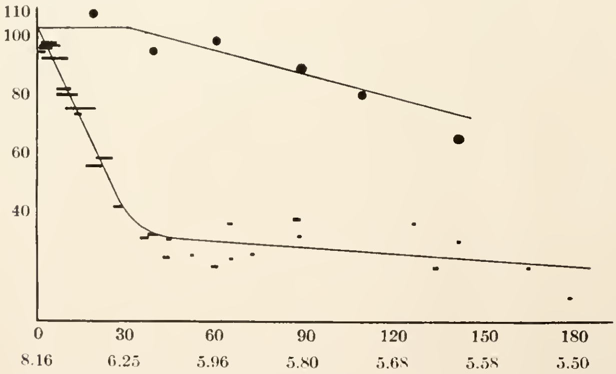

Fig. 3. The effect of carbon dioxide upon the oxygen consumption of fertilized *Arbacia* eggs.

Abscissa and ordinate as in Fig. 1.

— = carbon dioxide experiments.
● = hydrochloric acid experiments.

The average of the respiratory quotients obtained in air in thirty-two determinations was 0.71, which is lower than the average value of 0.78 obtained by Amberson (1928) in twenty experiments. The respiratory quotients in the presence of carbon dioxide must be interpreted with caution, for they range irregularly between 0.14 and 1.29. When the twenty-five carbon dioxide experiments in Table V are arranged in five groups of five experiments each, and the respiratory quotients and ratios of oxygen consumption and carbon dioxide production averaged, Table VI is obtained. It may be observed that, in the presence of carbon dioxide, the respiratory quotients are slightly higher than the control values up to 60 mm. Hg carbon dioxide tension. Above this tension, the respiratory quotients are lower. The carbon dioxide production and the oxygen consumption both decrease as the carbon dioxide tension increases.

TABLE VI

Summary of Experiments in Table V (Fertilized Arbacia Eggs)

Group	Experiments	CO_2 Tension mm. Hg	*RQ* Control	*RQ* CO_2 Tonometer	Ratio O_2 Consumption $\frac{CO_2 \text{ tonometer}}{\text{control}}$	Ratio CO_2 Production $\frac{CO_2 \text{ tonometer}}{\text{control}}$
I.....	5–9	1.6– 19.8	0.729	0.758	0.788	0.822
II....	10–14	16.2– 37.9	0.691	0.710	0.435	0.432
III...	15–19	37.8– 60.2	0.746	0.850	0.242	0.283
IV...	20–24	63.9– 88.0	0.734	0.546	0.282	0.208
V....	25–29	125.0–177.2	0.721	0.509	0.208	0.206

In order to determine whether carbon dioxide decreased the rate of oxygen consumption of fertilized *Arbacia* eggs by some internal effect, or merely by changing the pH of the sea water, a group of experiments was carried out in which approximately the same pH range was produced with hydrochloric acid which enters cells with difficulty, if at all. In these experiments, sea water was first made bicarbonate-free by adding concentrated hydrochloric acid to bring it to a pH of 3 to 4, and was ærated overnight. It was then returned to the desired pH by the addition of concentrated sodium hydroxide. The hydrogen ion concentration was determined by means of a quinhydrone electrode with an accuracy of 0.02 to 0.03 pH. The pH so obtained was compared with that of sea water at different carbon dioxide tensions as determined by Henderson and Cohn (1916).

The results of these experiments show a noticeable, but much smaller depression of the rate of oxygen consumption beginning at about pH 6.2 (corresponding to 35 mm. Hg carbon dioxide tension), and falling to about 62 per cent of the control at pH 5.6 (corresponding to that of sea water in equilibrium with carbon dioxide at 140 mm. Hg). (Table VII and Fig. 3.)

TABLE VII

The Effect of Hydrochloric Acid on the Respiration of Fertilized Arbacia Eggs

Experiment No.	Tonometer B pH	Resp. in Tonometer A			Resp. in Tonometer B			Ratio O_2 Consumption $\frac{B}{A}$
		O_2 Consumed	CO_2 Produced	*RQ*	O_2 Consumed	CO_2 Produced	*RQ*	
		cc.	*cc.*		*cc.*	*cc.*		
1	6.43	0.670	0.302	0.450	0.702	0.289	0.410	1.048
2	6.15	0.598	0.282	0.471	0.550	0.302	0.549	0.919
3	5.96	0.661	—	—	0.630	0.200	0.317	0.953
4	5.81	0.473	0.255	0.539	0.405	0.237	0.585	0.856
5	5.71	0.541	0.054	0.099	0.417	0.220	0.527	0.770
6	5.60	0.448	0.207	0.462	0.278	0.172	0.618	0.620

DISCUSSION

While the increased rate of oxygen consumption of *Paramecium* between carbon dioxide tensions of 15 and 66 mm. Hg probably lies within the experimental error, no experiment within this range resulted in a decreased rate of oxygen consumption. Since it is generally known that carbon dioxide causes an initial increase in the motility of *Paramecium* and other ciliates, followed by a decrease, it is suggested that the increased rate of oxygen consumption observed is a result of the increased motility of the organisms induced by carbon dioxide. This view is strengthened by the experiments performed upon *Arbacia* eggs in which there was no independent motility, and in which no increase in the rate of oxygen consumption was observed at any carbon dioxide tension.

Hydrochloric acid exerts a far less profound effect upon the rate of oxygen consumption of *Paramecium* and the fertilized eggs of *Arbacia* than does carbon dioxide at the same pH. Jacobs (1920*a*, 1920*b*, 1924) has shown that carbon dioxide far surpasses the acids which he studied in the rapidity with which its effects are produced. Hydrochloric acid, on the other hand, penetrates cells very slowly, if at all (Loeb, 1909; Jacobs, 1924). The differences in the results obtained by the action of carbon dioxide and of hydrochloric acid upon the rate of oxygen consumption of fertilized *Arbacia* eggs and *Paramecium* may perhaps be interpreted as due to differences in the ability of the two acids to penetrate the cell membrane, and to produce within the cell changes in pH, though it is, of course, possible that the relatively profound effect of carbon dioxide upon the rate of oxygen consumption of these cells may be due to some other specific effect of its molecule.

The respiratory quotients of *Paramecium* rise progressively as the carbon dioxide tension is increased (Table III). It is possible that the suppression of oxidations under these conditions results in the production of acid metabolites which drive out carbon dioxide from bicarbonate contained in the cells and in the surrounding medium. The respiratory quotients of the sea urchin eggs, however, do not increase in the presence of high carbon dioxide tensions (Table VI). In these cells it is possible that acid substances are not produced when the rate of oxygen consumption is decreased by carbon dioxide, or that such acid substances may be rapidly reconverted into a non-acid form so that they do not accumulate in appreciable amounts. It would appear, assuming the absence of gross errors, that the respiratory reactions of the two cells to carbon dioxide differ.

I wish to express my appreciation for the interest and stimulating suggestions made by Dr. W. R. Amberson, under whose direction these experiments were performed.

SUMMARY

1. The respiratory exchanges of *Paramecium caudatum* and the fertilized eggs of *Arbacia punctulata* have been studied by the method of gas analysis.

2. When *Paramecium* is exposed to different gas mixtures containing carbon dioxide at progressively increasing tensions, the rate of oxygen consumption increases slightly at the lower tensions and decreases at the higher tensions. The rate of oxygen consumption of fertilized *Arbacia* eggs is depressed by carbon dioxide at all of the tensions studied.

3. Moderate changes in the pH of the surrounding medium produced by hydrochloric acid have no apparent effect upon the rate of oxygen consumption of *Paramecium*. Similar changes in pH produced by hydrochloric acid diminish the rate of oxygen consumption of fertilized *Arbacia* eggs, although less markedly than does carbon dioxide at the same hydrogen ion concentration. The greater effectiveness of carbon dioxide in producing changes in the respiratory rate is presumably related to its greater penetrating power.

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A QUANTITATIVE STUDY OF THE EFFECT OF CARBON DIOXIDE UPON THE CLEAVAGE RATE OF THE ARBACIA EGG

CHARLOTTE HAYWOOD AND WALTER S. ROOT

(From the Marine Biological Laboratory, Woods Hole, Massachusetts, and the Department of Physiology, School of Medicine, University of Pennsylvania)

In two important respects carbon dioxide claims a place of peculiar interest for the physiologist. First, as a metabolic product it is present wherever there are active cells. Secondly, as many workers have observed, it has the effect of very promptly depressing certain cell activities. The exact extent of this depressant action of carbon dioxide upon the cells which produce it is an important question yet to be answered. A step in this direction, however, can be made by a determination, as quantitative as possible, of the effect of relatively low, accurately measured tensions of carbon dioxide upon some type of cell activity.

For such a study the fertilized egg of the sea urchin is especially useful, since it is less subject to wide variations than are many other cells, and since its rate of cleavage provides a convenient means of measuring any possible effects produced. Moreover, inasmuch as one of us (Root, 1930) has shown that the presence of carbon dioxide definitely limits the amount of oxygen consumed by the fertilized *Arbacia* egg, it is of interest to ascertain whether this effect of various known tensions of carbon dioxide upon cell oxidations is paralleled by its effect upon the cleavage rate.

Haywood (1927) has shown that, if carbon dioxide at a tension greater than 20 per cent of an atmosphere is added for even a few minutes to the sea water surrounding the newly fertilized eggs of *Arbacia,* cleavage suffers a distinct retardation. It might, therefore, be expected that lower tensions of the gas could also delay cleavage if administered for longer periods of time. In fact, this was earlier demonstrated by Smith and Clowes (1924) by a different method. These workers obtained the carbon dioxide from the bicarbonate of the sea water by the addition of hydrochloric acid, but with the result that the amount of bicarbonate remaining was not constant throughout this series of experiments. Since they themselves showed that the inhibitory effect of carbon dioxide is increased as the amount of bicarbonate is decreased, the results obtained by these workers are of a relatively complex nature.

In the present investigation, the newly fertilized eggs of *Arbacia* were subjected to sea water whose composition was not altered except by the addition of carbon dioxide. Moreover, we believe that our method has a further theoretical advantage because the data are based on the time required for one definite stage in the cleavage process to be reached (*i.e.*, the appearance of the first cleavage in 50 per cent of the eggs) rather than on the total number of cleavages occurring in all of the eggs in some arbitrarily selected time.

METHOD

Sea water containing the desired tension of carbon dioxide was prepared by equilibration with carbon dioxide-air mixtures, as described by Root (1930) elsewhere. This method consisted essentially of bubbling the gas from a mixing bottle into a tonometer containing sea water until the latter was found to be fully equilibrated with the gas, at which time duplicate gas samples were taken for subsequent analysis with a Bazett modification (1928) of the Haldane-Henderson gas analyzer. At the same time a tonometer for the control was equilibrated with room air. Our method was slightly modified from that of Root in two ways. First, both the carbon dioxide-air mixture and the room air used for equilibration were passed through a series of bottles containing sea water before entering the tonometers, to eliminate any possible change in osmotic pressure through evaporation. Secondly, the eggs were not added to the tonometers until *after* equilibration, because preliminary control experiments had indicated that the agitation of the eggs by the bubbling of the gas apparently had a retarding effect upon the rate of cleavage. Since only 0.9 cc. of egg suspension was introduced into 75–90 cc. of sea water in the tonometers which were used, it will be evident that any change in gaseous content due to the addition of the eggs was extremely small.

The lowering of the oxygen content of the carbon dioxide-air mixture caused by the addition of the carbon dioxide to room air could be entirely disregarded, for a previous study by Haywood (1927) has shown that the oxygen tension may be reduced far more than occurred in these experiments before the cleavage rate is affected by lack of oxygen.

The suspensions of eggs and sperm of *Arbacia punctulata* which were employed were freshly obtained and were freed of debris. A preliminary observation of their quality was made by fertilizing samples and noting the appearance of the fertilization membrane. The eggs were inseminated and then allowed to settle for five or six minutes. At the end of that time they were gently centrifuged by means of a hand centrifuge and the supernatant liquid containing sperm was removed, after which the egg suspension was diluted to the desired concentration by the addition of a small amount of sea water. At exactly 10 minutes after insemination, 0.9 cc. of the resulting egg suspension was introduced into each of the two tonometers, one of which was already equilibrated at the desired temperature with a carbon dioxide-air mixture, the other, with room air. Besides the two tonometers, a glass-stoppered bottle of about 80 cc. capacity, completely filled with sea water, also received 0.9 cc. of egg suspension. This bottle was useful as a check upon the control tonometer and also allowed inspection of the eggs at times when it was not desirable to open the control tonometer.

A temperature of 20.6° ± 0.5° C. was maintained by means of ice in all the experiments except those of eight hours' or more duration. In these longer experiments the variations in temperature were not more than ± 2.0° C.

After exposure to the gas of the tonometers for varying lengths of time, samples of the eggs were obtained for observation. After a preliminary precaution of cleaning the outlet tube of the tonometer with a stream of sea water, a few drops of the suspension were removed as a sample. This could be done without the admission of air by opening only the lower stop-cock while the tonometer was held in the hand for a moment, the warmth of the hand expanding the gas within the tonometer sufficiently to force out the sample. Further division of the eggs thus removed was immediately prevented by adding a weak solution of formaldehyde in sea water, which preserved them until a time when they could be accurately counted. It was, of course, necessary to take as few samples as possible in order to minimize the loss of gas from the tonometers. Four or five samples were usually sufficient to provide the requisite data, cleavage in 50 per cent of the eggs being completed in most cases before the third sample was taken. Since the samples could ordinarily be obtained within a few minutes of one another, the period during which the carbon dioxide tension was slightly lowered was a relatively short one. The maximum reduction in tension due to total sampling was never greater than 5 per cent.

The eggs were counted to determine the percentage showing the first cleavage. The counts were based upon samples of two hundred or more eggs, but occasionally it was necessary to use smaller samples. The percentages were then plotted against the time in minutes after insemination (Fig. 1). As already stated in an earlier paper (Haywood, 1927), the resulting curve theoretically represents the number of eggs which from minute to minute are undergoing the first cleavage, and from it by interpolation may easily be determined the time required for cleavage to appear in any given percentage of eggs. The time required for the first cleavage in 50 per cent of the eggs was chosen as a convenient criterion of the rapidity of the cleavage process, and will be referred to subsequently as the *cleavage time*. A comparison of the cleavage time of the eggs of the two tonometers, therefore, gives a basis for studying quantitatively the effect of carbon dioxide upon the rate of cleavage.

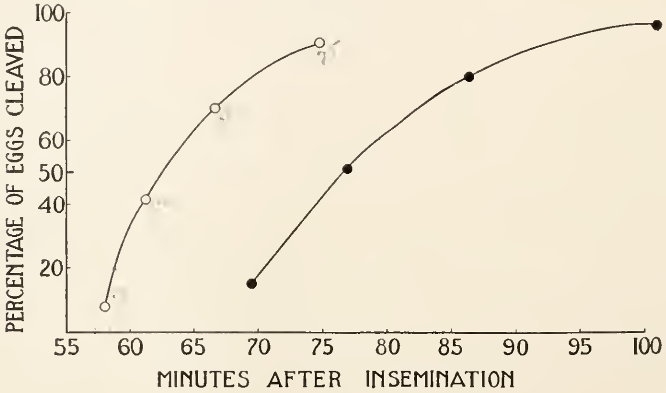

FIG. 1. The cleavage rate of *Arbacia* eggs during exposure to a carbon dioxide tension of 9.5 mm. Hg.

Abscissa = time, in minutes, after insemination.
Ordinate = percentage of eggs showing the first cleavage.
● = exposed eggs. ○ = control eggs.

Both sets of eggs were placed in the tonometers 10 minutes after insemination. Temperature = 20.6°–20.8° C.

RESULTS

The data of a typical experiment are shown graphically in Fig. 1. From the curve at the left, the cleavage time of the control was found to be 62.5 minutes, while a similar curve, unquestionably shifted toward the right, shows the effect of 9.5 mm. Hg tension of carbon dioxide in retarding the cleavage time to 76.5 minutes. This represents a delay of 14 minutes. Since exposure to carbon dioxide was not begun until 10 minutes after insemination, it must be realized that in every case the time of exposure was actually 10 minutes less than the time of cleavage. Thus, in the experiment cited, the 76.5 minutes measured as the cleavage time really represent but 66.5 minutes of exposure, and hence the value of 14 minutes given as the delay for the carbon dioxide tension employed may differ slightly from that which would have been obtained had the exposure been started immediately after fertilization. At the higher tensions of carbon dioxide, the curves obtained were somewhat flatter, and the number of eggs ultimately cleaving was often not as great. Abnormalities of cleavage were also to be found at some of the tensions employed. In such cases, the abnormally cleaved eggs were counted separately from the normal ones, but the total number of cleavages, abnormal as well as normal, was used in determining the cleavage time.

By means of sets of cleavage curves, obtained as were those in Fig. 1, the retardation of cleavage was determined for twenty-five different tensions of carbon dioxide, covering a range of from 1.8 mm. Hg to 44.0 mm. Hg. Some variation is naturally to be expected, especially since the range of carbon dioxide tensions employed was a relatively narrow one. Nevertheless, the general trend of the data is wholly obvious, as may be seen by observing Fig. 2. The pH values were taken from the data of Henderson and Cohn (1916). Tensions of as low as 4 mm. were able to retard cleavage 9 minutes, while one of 15 mm., which corresponds to about 2 per cent carbon dioxide, delayed it 16 minutes or more. With higher tensions of carbon dioxide, up to about 35 or 40 mm., the retardation was correspondingly greater. At tensions of 35 or 40 mm. and above, small increments of the gas were found to inhibit cleavage to a degree which was proportionally much greater than at lower tensions, and the curve, therefore, ascends steeply from this point on.

Whatever may be the importance of this critical tension of carbon dioxide, the effectiveness of very small amounts of the gas in retarding cleavage seems of real significance. Since tensions of the gas corresponding to 1 or 2 per cent—values which may conceivably approach carbon dioxide concentrations under conditions of crowding—are capable of causing an easily observed delay, it is apparent that the cells are highly sensitive to this gas.

The effects of carbon dioxide upon cell division appear quantitatively quite similar to those upon cell oxidations. This may readily be seen by comparing our curve in Fig. 2 with that obtained by one of us (Root, 1930) when the oxygen consumption of the eggs is plotted against the carbon dioxide tensions of the medium. It should be noted that both curves bend sharply at relatively the same carbon dioxide tension,—namely, at 35–40 mm. Hg. Apparently carbon dioxide acts upon some system within the cell which results in a depressed rate of oxygen consumption and an increased cleavage time.

As was mentioned earlier, when eggs were found to have divided abnormally in solutions containing carbon dioxide, the total number of cleavages, abnormal as well as normal, was used in plotting the cleavage curves. Therefore a different set of symbols was used in Fig. 2 to designate cleavage times in eggs where abnormalities resulted from exposure to the carbon dioxide. An examination of the relative number of abnormal and normal cleavages occurring at twelve different carbon dioxide tensions between 25.6 and 49.4 mm. Hg inclusive, showed the average ratio of abnormal to normal cleavages to be 2.5. The ratios ranged from 1 to 5.5 and tended to be somewhat higher for the higher carbon dioxide tensions.

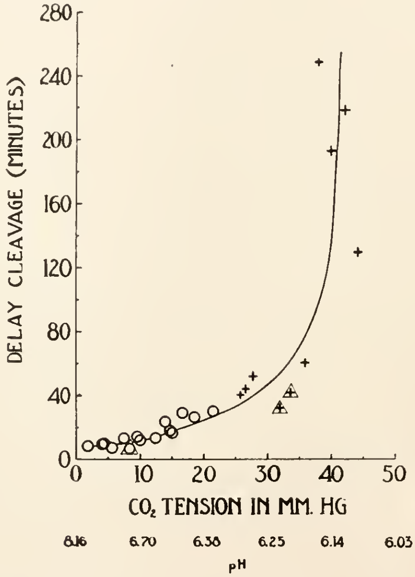

FIG. 2. The retardation of the first cleavage of *Arbacia* eggs during exposure to various carbon dioxide tensions.
Abscissa = carbon dioxide tension in mm. Hg; pH is also given.
Ordinate = minutes of retardation of the first cleavage.
○ = cleavages normal in at least 95 per cent of the cleaved eggs.
+ = abnormal cleavages included.
△ = values approximate only, since these were obtained by extrapolation.
Temperature = 20.6° ± 0.5° C.

After determining the extent to which cleavage was retarded by relatively low tensions of carbon dioxide, we next undertook to ascertain the tension of this gas which would be required to suppress cleavage completely. In these experiments, the eggs were exposed to carbon dioxide for eight hours or more, at the end of which time samples were taken and the percentage of cleaved eggs noted. Two controls were always carefully made in these experiments: First, a count was made of eggs which had been fixed in formaldehyde soon after fertilization, in order to make sure that no cleaved eggs were accidentally present; secondly, a count was made of eggs which had been kept for at least an hour in ordinary sea water, in order to determine whether or not the eggs used were fully capable of undergoing division. If the exposed eggs showed no cleavage in eight hours, the suppression of cleavage was regarded as complete. Such completely suppressed eggs were found to show no abnormalities in appearance, but retained completely the appearance of newly fertilized eggs. The results obtained are shown in Fig. 3, where the percentage of eggs cleaved is plotted against the tension of carbon dioxide used. Although variations in the results obtained with different groups of eggs make it impossible to designate any one tension of carbon dioxide as the threshold for the complete suppression of cleavage, the data are sufficiently consistent to allow a tentative estimate of 120 to 130 mm. Hg carbon dioxide for this value. This would correspond to 16 to 17 per cent carbon dioxide at 760 mm. Hg and 20.6° C., or a solution one-sixth saturated with carbon dioxide.

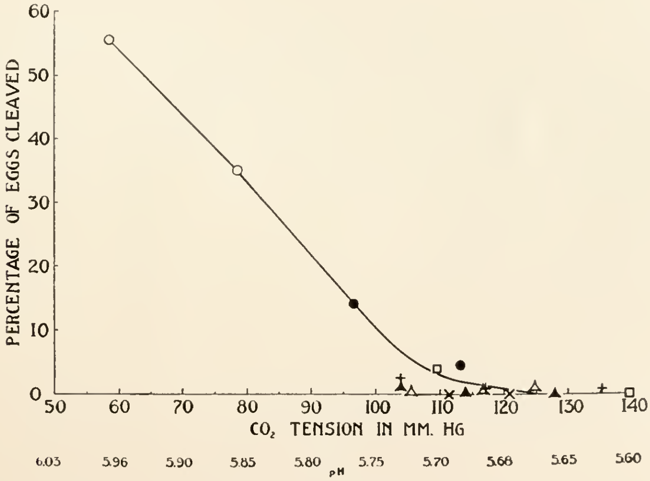

Fig. 3. The point of complete suppression of *Arbacia* eggs with carbon dioxide.

Abscissa = carbon dioxide tension in mm. Hg; pH is also given.

Ordinate = percentage of eggs showing the first cleavage after exposure of eight hours or more to carbon dioxide. Abnormals as well as normals are included.

The various symbols represent the different egg suspensions used.

Temperature = 20.6° ± 2.0° C.

This value of 16 to 17 per cent carbon dioxide, which is required for total inhibition of cleavage, is in wide disagreement with the 3.8 per cent obtained by Smith and Clowes (1924) at 20.0° C. It is important to realize, however, that the method of these investigators of liberating carbon dioxide from the bicarbonate of sea water by adding strong acid introduces a second variable—a varying bicarbonate content. These authors point out that, with a diminished bicarbonate content, the effect of carbon dioxide is more pronounced. The experimental conditions of Smith and Clowes' work differ sufficiently from those of ours to make difficult an agreement between the two sets of values.

SUMMARY

1. Sea water equilibrated with analyzed carbon dioxide-air mixtures was found to retard the first cleavage of *Arbacia* eggs which were introduced into the mixture ten minutes after insemination.

2. A measurable delay of cleavage occurred at tensions of carbon dioxide of as low as 4 mm. Hg. Retardation was progressively greater up to 120–130 mm. Hg, at which point complete suppression occurred.

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NOTES ON THE DEVELOPMENT OF FRAGMENTS OF THE FERTILIZED CHÆTOPTERUS EGG

EDMUND B. WILSON

(From the Marine Biological Laboratory, Woods Hole, and the Department of Zoölogy, Columbia University)

In a preceding paper (1929) it was shown that fertilized fragments of the unfertilized *Chætopterus* egg may segment exactly like whole eggs, at least as far as the 16-cell stage, and may develop into swimming dwarf larvæ that are sometimes closely similar to the normal whole trochophores. The experiments here reported were undertaken in the hope of obtaining light on progressive localization in this egg prior to cleavage by comparing the behavior of fragments of *fertilized* eggs taken at successive intervals during the maturation-fertilization period, somewhat as was long ago undertaken in the case of the nemertine egg by Yatsu (1904, 1910) and by Zeleny (1904). In this respect the results of numerous experiments have been disappointing; but some of the incidental ones may be suggestive of further work.[1] No report is here offered concerning the later stages of development; but it may be stated that a certain number of swimming dwarf larvæ are produced from the fragments, among them a few that approach the normal form. Thus far, however, none have been seen that were completely normal.

I

The experiments clearly show that up to the completion of maturation, the first cleavage of fragments of fertilized eggs does not differ in any very striking way from that of fertilized fragments of unfertilized eggs. In both alike a high percentage of the fragments divide unequally in nearly or quite the same proportions as in the development of whole eggs, producing 2-cell stages that appear as miniatures of the whole eggs at the same stage.[2] At the time of maturation a slight falling off of normal 2's is noticeable, owing to a corresponding rise in the percentage of abnormal cleavages; but it is uncertain whether this is significant. At any rate, the first marked change appears suddenly in fragments from eggs centrifuged at a critical period that begins about 10–15 minutes after extrusion of the second polocyte. At this time, as pointed out by Titlebaum (1928), the egg assumes a slightly marked pear-shape with the narrower end exactly at the upper pole.[3] Fragments produced by centrifuging the egg at this time show a sudden and marked drop in the percentage of normal first cleavages—often down to 10 per cent or less—and a corresponding rise in that of equal or sub-equal ones (Figs. 1–3), though a certain number of other ab-

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[1] The method of fragmentation was for the most part the same as in my preceding work (1929), namely, by centrifuging the eggs at high speed. In that work, by an error, the speed of rotation was stated to have been about 7,000 revolutions per minute; but this was too high. In the present case the speed, though not precisely determined, was not far from half that, and the time was reduced to 3–5 minutes. The proper speed and time for good fragmentation is easily determined by a few trials. A good many eggs were also cut in two individually by the glass hair; but the centrifuge method yields fragments in much greater numbers and of greater viability. Only eggs and sperm were used that showed in controls at least 98–100 per cent of normal 2-cell stages. The statistical results were obtained mainly from material fixed with dilute osmic acid, which when properly used gives perfect preservation of external form.

In these eggs, as is well known, maturation is initiated almost immediately after the eggs are set free into the sea-water, but proceeds no further until after fertilization. The times given vary more or less with temperature and other conditions.

TABLE I *

Fragments of Unfertilized and Fertilized Eggs

	Time of placing on centrifuge for 3–5 minutes	Total number of fragments	Normal 2-cell stages
1	Before fertilization	1350	1141 = 85 per cent.
2	Before fertilization	155	135 = 88 per cent.
3	12–13 minutes after fertilization	433	352 = 81 per cent.
4	25 minutes after fertilization, just before first polocyte	148	111 = 75 per cent.
5	2 minutes after first polocyte	306	207 = 68 per cent.
6	2 minutes after second polocyte	163	112 = 69 per cent.
7	10 minutes after second polocyte	155	6 = 4 per cent.

* Percentages in both tables are the nearest whole numbers. Nos. 2 and 7 are from different portions of the same lot of eggs.

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[2] Cf. Lillie (1909), Wilson (1929).

[3] This stage was long ago figured by Mead (1898) who showed, as I have also found, that the pear-shape appears during the approach and conjugation of the pro-nuclei and persists until shortly after the fusion-nucleus breaks down. Soon after the latter event the egg again becomes spheroidal, and then again pear-shaped, but now with the narrower end towards the lower pole. Immediately afterwards the polar lobe bulges forth at the lower pole and cleavage quickly follows, about 25–30 minutes after extrusion of the second polocyte.

normal forms also are commonly seen. These facts are in part shown in Table I. In the first line, for comparison, are shown the combined results of nine control experiments, with fertilized fragments of the unfertilized egg. Lines 3–7 give the results of a series of six experiments with fertilized eggs fragmented at successive intervals during the maturation-fertilization period. These results are typical of a much larger series except that the last item (No. 7) is selected as showing an especially low number (4 per cent) of normal 2's. As a control for this case, No. 2 shows a portion of the same batch of eggs, fragmented before fertilization, showing 88 per cent of normal 2's.

TABLE II †

(*a*) *Fertilized Fragments of Unfertilized Eggs*

	On centrifuge	Total number of fragments	Normal 2's	Equal or sub-equal	Too unequal	"3's"	4's
1	Before fertilization	234	214 = 90%	12 = 5%	8 = 3%	0	0

(*b*) *Fragments from a single batch of fertilized eggs placed for 5 minutes on the centrifuge at the same time (rather late pear, 19 minutes after first polocyte) and then fixed in osmic acid at successive intervals for examination. The first cleavage of the fragments occurred about eight and a half minutes after release from centrifuge.*

	Time of fixation	Total number	Normal 2's	Equal or sub-equal 2's	Too unequal	"3's"	4's
2	13½ minutes after removal from centrifuge	49	18 = 37%	27 = 55%	4 = 8%	0	0
3	18½ minutes after removal from centrifuge	100	33 = 33%	56 = 56%	10 = 10%	1	0
4	27 minutes after removal from centrifuge	98	18 = 18%	12 = 12%	3 = 3%	51 = 52%	14 = 14%
5	32 minutes after removal from centrifuge	98	10 = 10%	8 = 8%	4 = 4%	62 = 63%	14 = 14%

The percentages vary considerably from one experiment to another and hence are significant only within rather wide limits. Not the least doubt, however, can exist in regard to the sudden change that takes place at the critical period (No. 7); and it is equally certain that it is in large degree due to the replacement of unequal cleavages by equal or sub-equal ones (Figs. 1–3). These results fit exactly with Titlebaum's interesting discovery (1928) that whole eggs of *Chætopterus* and *Nereis*, when compressed transversely to the axis during the pear-shaped stage, often divide equally, and that the resulting 2-cell stages sometimes produce double embryos like those observed by Penners (1926) in Tubifex.[4]

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† It is probable that many of the forms listed as "3's" in the table are actually crossed 4's seen in foreshortened view, as explained in the text. It is possible, too, that most of the 2's remaining in line 5 may have stopped developing, as often happens in these cultures.

The facts are still more striking when account is taken of other forms of abnormal cleavage. A few of the fragments usually divide too unequally (Fig. 4), but with good eggs their number is always small (see Table II). Of greater interest are peculiar tripartite and quadripartite forms which closely resemble dispermic eggs (Figs. 5–9), but were finally found to arise mainly from the equal or sub-equal 2-cell stages, the second cleavage having already taken place because of delay in fixing the fragments for examination after their release from the centrifuge. It was also found that some of the tripartites arise simply by delayed division of one of the first two blastomeres, and that many others are actually 4-cell stages seen in foreshortened view, as explained below. Among the 4-cell stages are usually found a few that are normally formed (Fig. 6), as well as some that are nearly equal. In a high percentage of cases, however, and sometimes almost without exception, the 4-cell stages differ totally in aspect from the normal ones, consisting of two similar pairs of cells, each often more or less unequal, interlocking at right angles to each other to produce " crossed forms " which closely resemble those so often seen in dispermic eggs (compare Figs. 5, 6, 7). When viewed with low powers exactly in the axis of one pair, they may therefore readily be mistaken for 3-cell stages (Fig. 7).

These peculiar 4-cell forms undoubtedly arise from the 2's that precede them, and in large measure from the equal or sub-equal ones. This has been proved in a number of individual cases by following the second cleavage in life. It is shown on a much larger scale by fragmenting a large number of tested eggs from the same batch at the pear-shaped stage and dividing the products into several lots which are then fixed at successive intervals for later examination. It is obvious from the results, as partially shown in Table II, that the sudden drop in 2's and the simultaneous rise in " 3's " and 4's shown at lines

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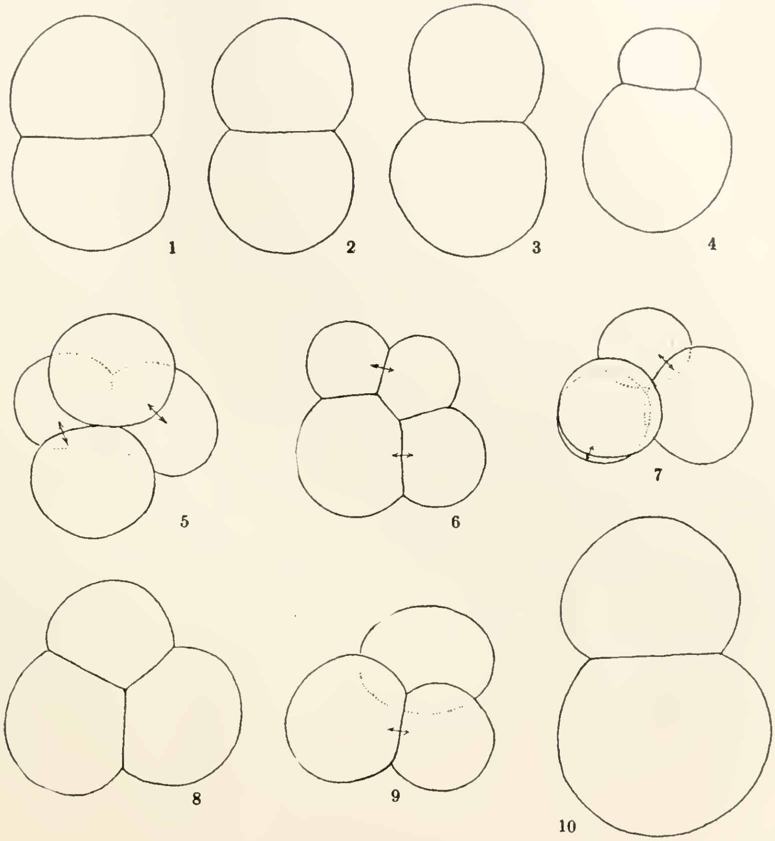
[4] In an unsuccessful attempt to duplicate Penners' result in the polychæte egg I repeatedly observed in 1926 equal first cleavage in the eggs of both *Chætopterus* and *Nereis* when segmenting under pressure; but no double embryos were obtained, nor did I recognize the critical stage as such.

4 and 5 is caused by occurrence at this time of the second cleavage in most of the fragments. The considerable drop in normal 2's at the same time indicates that some of them may have produced abnormal 4's (since normal 4's are rare or absent), but this point was not sufficiently examined. In any case, it is clear that a rather high percentage of "3's" and abnormal 4's must have come from the equal or subequal 2's. It would be interesting to make a more adequate statistical study of this point.



Figs. 1–10. Segmenting fragments of fertilized eggs fragmented at the pear-stage; 1–3, equal or sub-equal forms; 4, too unequal; 5, 7, crossed-type of 4-cell stage; 6, normal type; 8–9, 3-cell stages; 10, normal 2-cell stage from a whole egg. (All figures at the same enlargement.)

II

In the course of the foregoing experiments, my attention was directed to the fact that at certain stages the eggs of *Chætopterus* show a decided tendency to orient themselves while on the centrifuge. Lillie (1906) had previously found that when unfertilized eggs containing the first maturation spindle are centrifuged at a moderate speed (1,500–2,000 revolutions per minute), so as to elongate the egg without fragmentation, the polar spindle might afterwards be found in any position with reference to the secondary stratification but most frequently lies in the hemisphere containing the gray cap (oil-cap) that is formed at the central pole of elongation. Lillie attributed this to the fact that during maturation " the force of polarity has already caused a partial aggregation of the substances before the centrifugal force is applied, and the eggs, therefore, tend to rotate with the heavier pole in a distal direction " (p. 182). He concluded neverthelesss that " in a large percentage of the eggs, various causes combine to overcome this."

I have examined this question especially in eggs considerably elongated, but not fragmented, by centrifuging for a short time just after extrusion of the first polocyte, the latter subsequently affording an excellent landmark. The results show conclusively that in a large percentage of cases the polocyte is found lying either in or just outside the margin of the oil-cap that lies at the centripetal or central pole of elongation. Thus: out of 225 observed cases (4 experiments) placed on the centrifuge ½ to 1 minute after extrusion of the first polocyte, 155, or 70 per cent, showed the polocyte actually in the oil-cap, and 20, or 9 per cent, just outside its margin—*i.e.*, nearly 80 per cent in or very close to the oil-cap. This is especially noteworthy because in eggs thus elongated the oil-cap occupies but a relatively small area at the narrower end of the elongated egg. This, I think, leaves no doubt of a very marked tendency on the part of the eggs to orient themselves at this time with the animal pole turned towards the center of rotation.

Only a few other experiments were made with eggs at other stages. In one series, centrifuged immediately after extrusion of the second polocyte, 22 eggs showed 6 with the polocyte inside the oil-cap and 9 just outside it, *i.e.* nearly 70 per cent in or very near it. Experiments with eggs prior to maturation, though unsatisfactory for various reasons, point to the conclusion that the orientation is less marked than in later stages; but there are many sources of error in these cases. On the other hand, the results of centrifuging at the time of the first polocyte are so definite that this method should be useful for further experiments; for after elongation by short centrifuging the eggs may much more readily be cut in two individually and with less damage, and the plane of section may more readily be determined. Even in mass experiments at the time in question (and probably later), a rather high probability exists that fragments containing the oil-cap are from the upper hemisphere. By these methods it should readily be possible to settle the question, among others, whether both the upper and the lower hemispheres are capable of producing complete normal larvæ (as in *Cerebratulus*), or whether (as in *Dentalium*) only the lower hemisphere has such capacity.

Light on this question may perhaps be obtained in another way. It is a striking fact that after fragmentation of the fertilized eggs during the pear-shaped stage most of the fragments that segment, and sometimes very nearly all, are the hyaline or lightly granular ones. These fragments rarely if ever divide with a polar lobe, while the small number of dark granular ones that divide commonly form such a structure. This indicates that the clear fragments are for the most part from the upper hemisphere (as might be expected also from the position of the amphiaster in the normal whole eggs), though they rarely bear polocytes. All the facts are explained under the supposition that the eggs orient themselves to a considerable extent while on the centrifuge, and hence are commonly fragmented across the egg-axis in such a manner as to separate the lower, granular region (in which presumably lies the lower pole-plasm or its homologue) from the upper one containing the amphiaster and chromosomes. The frequent absence of the polocytes from the latter fragments may very well be due to the circumstance that in continued centrifuging at fairly high speeds the region of the oil-cap at the light pole is almost invariably the first to separate from the egg, in the form of a small spheroidal fragment. By this process—which I have repeatedly observed—the polocytes would be carried off with the oil-cap in a large percentage of cases. There are here interesting possibilities for further experiment.

III

The foregoing observations do not, I fear, throw much light on the problems of localization in the *Chætopterus* egg. At first sight it may seem that the sudden change in the form of cleavage that appears in both whole eggs and their fragments after centrifuging at the pear-shaped period points to the occurrence of a corresponding crisis in the localizing process at that time—such a possibility is certainly suggested by the facts in the leeches (Whitman, Schleip) and oligochæts (Vejdovský, Penners) where the substance of the polar rings or pole-plasms is segregated, and in some measure localized, during and after the extrusion of the polocytes. It is this material—in some cases contained in the polar lobe (*Myzostoma*, *Ilyanassa*, *Dentalium*)—which during the first cleavage passes into the posterior or CD-blastomere and thus marks out the bilaterality and antero-posterior differentiation of the embryo. Passing later, wholly or in part, into the somatoblasts (2d and 4d) it ultimately enters the teloblasts that play so important a part in building the body. In the absence of this material—*e.g.*, after removal of the polar lobe (*Ilyanassa, Dentalium*), or of the lower polar area (*Dentalium*), or in case of the isolated AB-blastomere—*Lanice, Dentalium, Tubifex*)— the embryo is unable to produce a perfect larva or, it would seem, one that is bilaterally symmetrical.[5] In the case of *Chætopterus,* however, there is as yet nothing to show whether segregation of the material in question takes place during the pear-shaped period or earlier. The latter alternative is suggested by Lillie's cytological observations on the ovarian egg (1906), by the formation of a polar lobe during the first cleavage, and by the conditions observed in *Myzostoma* and *Dentalium*. Uncertainty in regard to this point is, however, caused by the strange fact, determined by both Whitaker and myself and confirmed by my more recent observation, that after horizontal section of the unfertilized egg and subsequent fertilization, only the lower or vegetative fragment divides with a polar lobe, yet both fragments alike divide unequally and in nearly the same proportions as in case of the whole egg. Without an explanation of this puzzling fact the problem of localization in the *Chætopterus* egg will remain unsolved.

Turning aside from this question, it seems to me that the strong tendency towards equal cleavage, shown alike by whole eggs and their fragments after centrifuging at the pear-shaped stage, gives little or no indication as to whether localizing processes are in progress at the time. That tendency may most simply be regarded as a merely mechanical effect of centrifuging, similar in principle to that suggested by Titlebaum (1928) in explanation of the corresponding effect produced on whole eggs by lateral compression at the same stage. It is at this time, as is clearly seen in Mead's figures (1898) that the pronuclei are conjugating and the sperm-asters are at their maximum development (though the definitive cleavage-spindle is not fully established until a little later). Strong centrifuging at this period may simply produce a dislocation of the developing amphiaster from its normal adjustment to the organization of the cytoplasm existing at the time, more specifically, perhaps, its relation to a regional segregation of material corresponding to the pole-plasms of the leeches and oligochæts. The amphiaster, being thus liberated from its normal connections, is left free to develop as a symmetrical or homopolar structure, such as it seems to be during the earlier period of its development, thus leading to equal cleavage.[6]

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[5] For the experimental proof of this, see Crampton (1896), Wilson (1904a, 1904c), Penners (1926).

The nature of the later development might be expected to depend on the position that the amphiaster happens to take with respect to the material in question—if the latter is equally divided, symmetrical twinning might be expected; if unequally divided, various types of asymmetrical twinning might result, such, for instance, as those long ago described in earth-worms by Kleinenberg (1879) and Vejdovský (1888–1892). One is tempted to seek in the same direction some sort of clue to the peculiar 4-cell crossed type which so commonly follows equal first cleavage, and which differs in so marked and definite a way from the normal 4's (Figs. 5–7). It seems fairly clear that in the eggs of *Tubifex, Chætopterus* and *Nereis* the tendency to twinning shown after equal first cleavage involves some kind of release of the first two cells from the integrating factors which in the normal development are responsible for the production of a single embryo. Such a release, conceivably, might leave the two resulting cell-pairs free to seek the position of least mechanical resistance, *i.e.*, that of minimal contact area, which is I think that of the crossed form.

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[6] See the accurate figures of Mead (1898) which, through his courtesy, I have recently had an opportunity to compare with the original preparations. See also the corresponding accounts of Vejdovský (1888–92), of Vejdovský and Mrázek (1903), on *Rhynchelmis*, and those of Schleip (1914) on *Clepsine*.

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STUDIES OF PHOTODYNAMIC ACTION

II. The Relationship between Hemolysis by Irradiated and Non-Irradiated Eosine

HAROLD F. BLUM

Department of Physiology, Harvard Medical School

Many photodynamic substances when in sufficient concentration bring about changes in cells which are similar to the changes produced by the irradiated dye in less concentration. This effect was studied very early by Tappeiner and Jodlbauer (1904), Jodlbauer and Busck (1905) and others. Sacharoff and Sachs described hemolysis by non-irradiated erythrosine and other photodynamic substances in the paper in which they first described photodynamic hemolysis (1905). Jodlbauer and Haffner (1921*a*) summarize a certain number of such studies and point out that among photodynamic substances, those which bring about hemolysis in the lowest concentration when not irradiated are generally the most active in bringing about photodynamic phenomena in living cells. Likewise, they are most active in flocculating the colloids of hemolyzed blood cells. While this generalization has many exceptions, it indicates a correlation between the action of irradiated and non-irradiated photodynamic substances.

Jodlbauer and Haffner (1921*b*) studied the effect of hydrogen ion concentration on hemolysis by non-irradiated eosine and rose bengale (tetra-brom tetra-iodo fluorescein). They found that the hemolytic effect, as measured by the minimum dye concentration necessary to produce hemolysis, was at a minimum in the region of neutrality and increased as the reaction became either more acid or more alkaline. This increase was more pronounced on the acid side. In acid solutions of dye in high concentrations, fixation was found to take place instead of hemolysis. The fixing action, like the hemolytic action, increased with increasing acidity. These investigators did not examine the effect of hydrogen ion concentration or dye concentration on the hemolytic and fixing activity of the irradiated dyes. This has been done in the experiments described below, the results of which point to another correlation between the action of irradiated and non-irradiated dyes, namely, that the hemolytic and fixing action of the irradiated dye is modified in the same way by hydrogen ion concentration as is that of the non-irradiated dye.

EXPERIMENTAL

Effect of Hydrogen Ion Concentration on Hemolysis and Fixation by Irradiated Eosine.—In a preceding paper (Blum, 1930), it was shown that previously irradiated eosine may bring about hemolysis to a degree less than that occurring when the dye is irradiated together with the cells. Therefore, parallel series of experiments were conducted under both conditions together with a like series, using the non-irradiated dye. The technique of the experiments was the same as that described in the above paper. A much narrower range of hydrogen ion concentration was studied than that examined by Jodlbauer and Haffner (1921*b*), as it seemed wise to avoid as much as possible the hemolytic effects of the hydrogen and hydroxyl ions themselves. Within the range here used, pH 6.0 to pH 7.7, no great difference in cell volumes occurs in

TABLE I

Effect of Hydrogen Ion Concentration on Hemolytic Action of Irradiated and Non-Irradiated Eosine.

All solutions contain sodium phosphate buffer, isosmotic with 0.15 M NaCl. Observations made after 7 hours in dark following irradiation. *H* = complete hemolysis; (*H*) = partial hemolysis; *a* = cells plus eosine irradiated; *b* = eosine irradiated alone, cells added immediately after exposure; *c* = non-irradiated. *a* and *b* irradiated 1 hour (11:00 A.M.–12:00 M., Sept. 15, 1929).

Concentration of Eosine *per cent*	pH 6.0			pH 6.4			pH 6.9			pH 7.3			pH 7.6		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
1.0 (0.0014 M)	—	—	—	—	*H*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	(*H*)
0.5 (0.0007 M)	—	—	—	(*H*)	*H*	(*H*)	(*H*)	*H*	(*H*)	(*H*)	*H*	—	(*H*)	(*H*)	—
0.25 (0.00035 M)	(*H*)	—	—	*H*	*H*	(*H*)	*H*	*H*	—	(*H*)	*H*	—	(*H*)	(*H*)	—
0.125 (0.00017 M)	(*H*)	*H*	—	*H*	*H*	(*H*)	*H*	*H*	—	(*H*)	(*H*)	—	(*H*)	*H*	—
0.062 (0.00009 M)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	*H*	—
0.031 (0.00004 M)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	*H*	—
0.015 (0.00002 M)	*H*	(*H*)	—	*H*	*H*	—	*H*	(*H*)	—	*H*	(*H*)	—	*H*	(*H*)	—
0.007 (0.00001 M)	*H*	(*H*)	—	*H*	(*H*)	—	*H*	—	—	*H*	(*H*)	—	*H*	(*H*)	—
0.004 (0.000005 M)	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.002 (0.000002 M)	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

the solutions used.[1] Thus we may assume that the hemolytic action of hydrogen and hydroxyl ions is practically negligible in our experiments.

Three like series of dilutions of the dye were used at each hydrogen ion concentration; (*a*) dye irradiated together with cells, (*b*) dye irradiated alone, cells added subsequently in the dark and (*c*) dye not irradiated. Tables I and II are taken from typical experiments covering overlapping regions of eosine concentration, and do not agree absolutely in a quantitative sense as regards the irradiated dye. The conditions of irradiation never being the same in any two experiments, absolute agreement is never obtained, but each experiment performed demonstrates certain qualitative differences between the irradiated and non-irradiated dye. A certain parallelism may be observed in the action of the irradiated and non-irradiated dye in that, generally speaking, hemolysis occurs most readily in both cases at the same hydrogen ion concentrations; the irradiated solutions, however, show hemolysis at lower dye concentrations. The solutions irradiated alone (*b*) always appear to take a place intermediate between the non-irradiated dye (*c*) and the dye irradiated with the cells (*a*) as regards their hemolytic activity. The explanation of this latter fact has been discussed by the writer (1930).

TABLE II

Effect of Hydrogen Ion Concentration on Hemolytic Action of Irradiated and Non-Irradiated Eosine.

All solutions contain sodium phosphate buffer, isosmotic with 0.15 M NaCl. Observations made after 6 hours in dark following irradiation. *P* = precipitate following observation of complete hemolysis at an earlier time. Other symbols as in Table I. *a* and *b* irradiated 1 hour and 15 minutes (1:45–3:00 P.M., May 10, 1929).

Concentration of Eosine	pH 6.0			pH 6.5			pH 7.0			pH 7.4			pH 7.7		
per cent	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
4.0	—	—	—				—	*P*	*P*				*H*	*H*	*H*
2.0				—	—	—				*H*	*H*	*H*			
1.0	—	—	—				—	(*H*)	*H*				(*H*)	*H*	(*H*)
0.5				—	*H*	*H*				(*H*)	(*H*)	—			
0.25	—	—	—				(*H*)	—	—				—	(*H*)	—
0.125				(*H*)	(*H*)	—				(*H*)	—	—			
0.062	—	—	—				(*H*)	—	—				(*H*)	—	—
0.031				(*H*)	—	—				(*H*)	—	—			
0.015	—	—	—				(*H*)	—	—				(*H*)	—	—
0.007				*H*	—	—				(*H*)	—	—			

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[1] See Blum (1930).

We note in both Tables I and II, at acid reactions, that hemolysis occurs in higher concentrations in non-irradiated than in irradiated solutions. Here again series *b* appears to be intermediate between *a* and *c*. This apparently paradoxical behavior is due to fixation of the cells, as described by Jodlbauer and Haffner (1921*b*) for high concentrations of non-irradiated eosine and rose bengale at acid reactions. This is demonstrated in the following type of experiment which was carried out in the usual manner, except that after several hours in the dark following irradiation the cells were examined for fixation by treatment with distilled water. The solutions were pipetted off from all those tubes in which complete hemolysis had not occurred, leaving only the cells and debris in the bottom of the tubes. The solutions were then replaced by distilled water and allowed to stand for several hours. At the end of this time, the tubes were again examined for hemolysis, the determinations with the naked eye being checked by microscopic examination. The cells in those tubes which did not show hemolysis before or after this treatment were considered as completely fixed and are designated in Table III by the letter *F*. Those tubes which showed some hemolysis, but in which intact cells could be observed, are represented by (*F*) to indicate partial fixation of the cells. Partial fixation was also considered to have occurred in those tubes which showed hemolysis before treatment with distilled water but no hemolysis after, and these are likewise designated by (*F*). Partial hemolysis before this treatment is designated by (*H*), and complete hemolysis by *H* as in the previous tables.

This treatment of the cells is rather severe and selection of a criterion of fixation is rather arbitrary. Nevertheless, the results demonstrate in a striking manner that fixation, like hemolysis, is shifted into a lower concentration of the dye by irradiation. They also show that fixation is most apparent in both irradiated and non-irradiated dye at the same hydrogen ion concentration, and that series *b* is again intermediate between *a* and *c* as in the case of hemolysis. A consideration of the data in Table III indicates the fallacy of adopting hemolysis as a quantitative measure of photodynamic action. Very false deductions may be based upon the simple observation that hemolysis does not occur under a given set of conditions, since this may indicate either that the action of the photodynamic substance is too weak to produce hemolysis, or that it has produced fixation instead. We are obviously dealing with two manifestations of the action of eosine on red blood cells which are both modified in much the same way by irradiation and by hydrogen ion concentration.

Still a third manifestation of the action of non-irradiated and irradiated dyes appears in the precipitation of cell constituents from suspensions of hemolyzed blood cells. Jodlbauer and Haffner (1921*b*) have described flocculation of hemolyzed cells by non-irradiated eosine and rose bengale. Table IV shows the results of an experiment which affords a comparison between the action of irradiated and non-irradiated eosine on hemolyzed cells. This experiment was conducted simultaneously with the one described in Table II and under exactly the same conditions, except that blood cells hemolyzed with distilled water were used instead of intact blood cells. It may be readily seen that precipitation, like hemolysis and fixation, is more pronounced in irradiated than in non-irradiated solutions of the dye, and that series *b* is intermediate between *a* and *c*. Again, as with hemolysis and fixation, precipitation is most pronounced at the same hydrogen ion concentrations in both the irradiated and the non-irradiated dye.

Table III

Fixation and Hemolysis of Red Blood Cells by Irradiated and Non-Irradiated Eosine.

All solutions contain sodium phosphate buffer, isosmotic with 0.15 M NaCl. Observations made after 7 hours and 30 minutes in dark following irradiation. *F* = complete fixation; (*F*) = partial fixation. Other symbols as in Tables I and II. *a* and *b* irradiated 2 hours and 15 minutes (October 11, 1929).

Concentration of Eosine	pH 6.0			pH 6.5		
per cent	*a*	*b*	*c*	*a*	*b*	*c*
2.0	*F*	*F*	*F*	(*F*)	(*H*) (*F*)	(*F*)
1.33	*F*	*F*	*F*	(*H*) (*F*)	(*H*)	(*H*)
0.89	*F*	*F*	*F*	(*H*) (*F*)	*H*	*H*
0.59	*F*	*F*	(*F*)	(*H*) (*F*)	*H*	(*H*)
0.39	*F*	(*H*) (*F*)	(*F*)	(*H*)	*H*	(*H*)
0.26	*F*	*H*	—	(*H*)	*H*	—
0.17	*F*	*H*	—	(*H*)	*H*	—
0.11	(*H*)	*H*	—	(*H*)	*H*	—
0.075	(*H*)	*H*	—	(*H*)	*H*	—
0.050	(*H*)	*H*	—	(*H*)	*H*	—
0.	—	—	—	—	—	—

Table V is a record from the experiment described in Table I but represents observations made five hours later or after twelve hours in the dark following irradiation. This table is inserted to show how precipitation may mask the occurrence of hemolysis, and to emphasize the importance of making more observations than one in the course of a single experiment. Tubes often show partial hemolysis at one period with subsequent precipitation of the hemolyzed cell constituents which may mask the presence of unhemolyzed fixed cells.

TABLE IV

Effect of Hydrogen Ion Concentration on Precipitation of Hemolyzed Cell Constituents by Irradiated and Non-Irradiated Eosine.

Symbols as in preceding tables. P = precipitate. a and b irradiated 1 hour and 15 minutes (1:45–3:00 P.M., May 10, 1929). All solutions contain sodium phosphate buffer isosmotic with 0.15 M NaCl. Observations made after 6 hours in dark following exposure of a and b.

Concentration of Eosine	pH 6.0			pH 6.5			pH 7.0			pH 7.4			pH 7.7		
per cent	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
4.0	*P*	*P*	*P*				*P*	*P*	*P*				—	—	—
2.0				*P*	*P*	—				slight *P*	—	—			
1.0	*P*	*P*	*P*				*P*	*P*	—				—	—	—
0.5				*P*	*P*	—				—	—	—			
0.25	*P*	*P*	slight *P*				*P*	*P*	—				—	—	—
0.125				—	—	—				—	—	—			
0.062	*P*	trace *P*	—				—	—	—				—	—	—
0.031				—	—	—				—	—	—			
0.015	*P*	—	—				—	—	—				—	—	—
0.007				*P*	—	—				—	—	—			

TABLE V

Effect of Hydrogen Ion Concentration on Hemolytic Activity of Irradiated and Non-Irradiated Eosine.

Same experiment described in Table I. Observations made after 12 hours in dark following irradiation. Symbols as in preceding tables.

Concentration of Eosine	pH 6.0			pH 6.4			pH 6.9			pH 7.3			pH 7.6		
per cent	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
1.0	*P*	*P*	—	*P*	*H*	*P*	*P*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	(*H*)
0.5	*P*	*P*	—	*P*	*H*	(*H*)	*P*	*H*	(*H*)	*H*	*H*	(*H*)	*H*	*H*	(*H*)
0.25	*P*	*P*	—	*H*	*H*	(*H*)	*P*	*H*	—	*H*	*H*	—	*H*	*H*	—
0.125	*P*	*P*	—	*H*	*H*	(*H*)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—
0.062	*P*	*P*	—	*H*	*H*	(*H*)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—
0.031	*P*	*P*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	*H*	—
0.015	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	(*H*)	—
0.007	*H*	(*H*)	—	*H*	(*H*)	—	*H*	—	—	*H*	(*H*)	—	*H*	(*H*)	—
0.004	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.002	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Changes in Hydrogen Ion Concentration of Eosine Solutions during Irradiation.—Since hydrogen ion concentration exerts a marked effect on the hemolytic action of both irradiated and non-irradiated eosine, changes in hydrogen ion concentration during irradiation of unbuffered solutions might be expected to markedly affect the hemolytic activity of such solutions. Such changes in hydrogen ion concentration during the course of irradiation may be readily demonstrated in dilute unbuffered solutions of eosine by means of indicators. In 0.0005 M eosine solution, the sulphonthalein indicators may be used with sufficient accuracy to observe differences of hydrogen ion concentration corresponding to 0.5 pH. The procedure in such determinations was to add the indicators to samples of irradiated and non-irradiated dye, and to compare these to buffered solutions containing the same concentration of dye and indicator. The shift of hydrogen ion concentration during irradiation was always found to be toward the acid side. In one set of experiments, unbuffered 0.0005 M eosine solutions which had a hydrogen ion concentration of approximately pH 6.5 before irradiation were found to have increased in hydrogen ion concentration to approximately pH 4.0 in the course of five hours' irradiation. The latter value is far outside the range of hydrogen ion concentrations examined in the above experiments.

Since most of the experiments of previous investigators of photodynamic processes have been carried out in unbuffered solutions, it is quite probable that such change in hydrogen ion concentration may have been a very important factor in the results obtained. It is probable that some of the failures of other investigators to produce hemolysis by previously irradiated photodynamic substances, as described by the writer (1930), may have been due to the shifting of the hydrogen ion concentration into an acid region in which fixation and not hemolysis occurs with the concentration of the dye used. The writer has, in fact, observed fixation of cells added to previously irradiated unbuffered solutions of eosine. This criticism applies to the experiments of Sacharoff and Sachs (1905), Hausmann (1909 and 1910) and Hasselbach (1909), who were unable to demonstrate hemolysis with previously irradiated solutions of numerous photodynamic substances.

Hemolysis and Fixation by Hydrogen Peroxide and Non-irradiated Eosine.—In the preceding paper of this series (Blum 1930), the probability was discussed that the alteration in hemolytic activity of eosine by irradiation is due to an increase in oxidizing power of the dye solution, resulting in the oxidation of cell constituents. From the experiments described above it appears that the effect of irradiation is simply to increase the effects produced by non-irradiated eosine, evidenced by the shifting of hemolysis, fixation, and precipitation into lower concentrations of the dye. This suggests that the action of the non-irradiated eosine is also an oxidation which is increased by irradiation. This is probably not the case, however, since the non-irradiated dye is not capable of oxidizing a readily oxidizable substance such as the iodide ion. Furthermore, at least certain of the changes produced by the non-irradiated dyes may be readily reversed by simply washing out the dye, (Kudo and Jodlbauer, 1908). A possible explanation of the similarity of action between irradiated and non-irradiated eosine is that irradiation brings about oxidative changes which are simply superimposed upon changes brought about by the non-irradiated dye. This assumption need not take into consideration the mechanism of the changes brought about by the non-irradiated dye.

Such an assumption can be tested to a certain extent by finding whether oxidation alone may bring about hemolysis, and whether the effect of this oxidation is increased by the presence of non-irradiated eosine. Hydrogen peroxide is capable of oxidizing iodide ion and must, therefore, have a greater oxidizing power than non-irradiated eosine. The following experiment shows the result of treating red blood cells with hydrogen peroxide and with hydrogen peroxide plus non-irradiated eosine. Series of dilutions of hydrogen peroxide and of eosine were used. The hydrogen peroxide solutions were freshly prepared from " Superoxol " (Merck) containing 30 per cent hydrogen peroxide, which was neutralized with 0.1 N sodium hydroxide and diluted with the usual mixture of primary and secondary sodium phosphates. The greatest concentration of hydrogen peroxide used in the experiments was 3 per cent, which represents a dilution of salt content of approximately 10 per cent. Much greater dilutions with water are necessary to bring about hemolysis, and as a matter of fact fixation and not hemolysis occurs at this concentration of hydrogen peroxide. In the lower dilutions of hydrogen peroxide which were used, the reduction of salt content is certainly negligible. The technique of preparation and addition of blood cells was the same as described for previous experiments. Table VI shows the result of an experiment in which the hydrogen ion concentration of the solutions was buffered at pH 6.0. It will be noted that with the peroxide solutions containing no eosine, hemolysis occurs at concentrations of 0.37 per cent to 1.5 per cent inclusive, while above this at 3 per cent fixation occurs. At the latter concentration the hemoglobin is changed to a brownish substance, probably methemoglobin. This confirms the findings of both Schmidt and Norman (1922) and Rigoni (1926). The former found that no hemolysis took place in cells treated with hydrogen peroxide (concen-

TABLE VI

Hemolysis and Fixation of Cells by Hydrogen Peroxide and Non-Irradiated Eosine

Symbols as in Table III. All solutions contain sodium phosphate buffer pH 6.0, isosmotic with 0.15 M NaCl. Observations made six hours after addition of blood cells to eosine- peroxide mixtures.

Eosine	H_2O_2										
	3.0% (0.9 M)	1.5% (0.45 M)	0.75% (0.22 M)	0.37% (0.11 M)	0.19% (0.05 M)	0.09% (0.03 M)	0.05% (0.015 M)	0.02% (0.007 M)	0.01% (0.004 M)	0.005% (0.002 M)	0.%
per cent 1.0 (0.0014 M)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
0.5 (0.0007 M)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
0.25 (0.00035 M)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	(*F*)	—	—	—
0.125 (0.00017 M)	*F*	*F*	*F*	*F*	*F*	(*H*) (*F*)	—	—	—	—	—
0.062 (0.00009 M)	*F*	*F*	*F*	(*H*) (*F*)	(*H*) (*F*)	—	—	—	—	—	—
0.031 (0.00004 M)	*F*	*F*	(*H*) (*F*)	*H*	*H*	(*H*)	(*H*)	—	—	—	—
0.015 (0.00002 M)	*F*	(*H*) (*F*)	*H*	*H*	*H*	*H*	(*H*)	—	—	—	—
0.007 (0.00001 M)	*F*	(*H*)	(*H*)	*H*	*H*	*H*	*H*	(*H*)	(*H*)	(*H*)	—
0.004 (0.000005 M)	*F*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	—	(*H*)	—
0.002 (0.000002 M)	*F*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	—	—	—
0.	*F*	*H*	*H*	(*H*)	—	—	—	—	—	—	—

tration not stated) but that methemoglobin formation occurred. The latter found hemolysis in only a given range of hydrogen ion concentrations, stronger as well as weaker solutions failing to bring it about. We see, thus, that an oxidizing agent alone may produce both hemolysis and fixation.

Further examination of Table VI shows that the addition of non-irradiated eosine to the peroxide greatly alters the region of the concentration at which hemolysis and fixation occur. The presence of the dye shifts the region of hemolysis and that of fixation into much lower concentrations of peroxide. Likewise the presence of peroxide shifts these phenomena into lower eosine concentration. Thus, peroxide plus eosine may accomplish hemolysis or fixation in concentrations at which neither alone is effective.

Treatment with hydrogen peroxide plus non-irradiated eosine markedly alters the solubility of hemolyzed blood cell constituents. Decrease of solubility is evidenced by precipitates varying from slight cloudiness to heavy flocculation. While the results at hand demonstrate a definite decrease in solubility at certain concentrations of hydrogen peroxide plus eosine as compared to the same concentration of eosine alone, a more careful analysis does not seem justifiable without further data.

From these experiments it seems clear that the hemolyzing and fixing effect of non-irradiated eosine may be increased by the action of an oxidizing agent such as hydrogen peroxide. We see also that oxidation alone may produce hemolysis and fixation. This evidence seems to support our assumption that the oxidations brought about by the irradiation of the dye are merely superimposed upon the action of the non-irradiated dye. Evidence was offered by the writer (1930) that in the presence of potassium iodide, irradiated eosine returns to its non-irradiated form after having oxidized a corresponding quantity of iodide ion. Similarly, we must assume that if an oxidation of cell constituents takes place, the irradiated dye must return to its non-irradiated form after having oxidized these substances. Thus we may consider the dye as playing a dual rôle, first as the non-irradiated dye, and secondly as an oxidizing agent produced by irradiation.

As shown in the above paper, the oxidizing power of previously irradiated eosine is never greater than the molecular equivalency of the dye. Consequently, we should expect that if the irradiated eosine has an oxidizing power of the same order as hydrogen peroxide, the two substances should bring about hemolysis in approximately the same concentration if the effect of the non-irradiated eosine is neglected. On the basis of the above hypothesis the hemolytic effect of the previously irradiated eosine is a summation of the oxidation and the effect of the non-irradiated dye, and hence, we should compare the irradiated eosine to peroxide plus an equal concentration of the non-irradiated dye. Comparison of Tables I and VI shows, however, that the concentrations of hydrogen peroxide required to produce hemolysis are many times greater than the concentrations of previously irradiated eosine which produce hemolysis under comparable conditions. Table I shows that at pH 6.0, previously irradiated eosine brings about hemolysis in concentrations as low as 0.00001 M. On the other hand, Table VI shows that, at the same hydrogen ion concentration, 0.002 M hydrogen peroxide is required to bring about partial hemolysis in the presence of 0.00001 M non-irradiated eosine. At slightly lower peroxide concentrations, hemolysis no longer occurs in any eosine concentration which alone does not produce hemolysis. It would thus appear that irradiated eosine is actually a much more powerful oxidizing agent than hydrogen peroxide. Noack (1920) has found, similarly, that irradiated eosine was more effective in oxidizing the sap of *Aloe soccotrina* than was hydrogen peroxide.

DISCUSSION

The correlation made by Jodlbauer and Haffner (1921*a*) between the photodynamic activity of substances and their ability to hemolyze red blood cells in the dark, is readily explained on the basis of the hypothesis outlined above. Since the total observable effect produced by irradiated dyes would be a summation of the effect of the non-irradiated dye and the oxidative changes brought about concomitant with irradiation, an increase in the first of these factors would result in an increase of the total effect. Thus, other factors being equal, the substances having the greatest hemolytic activity in the non-irradiated state should also show the greatest hemolytic effect when irradiated.

Jodlbauer (1926) suggests a quite different explanation of this correlation. He assumes that photodynamic action is dependent upon adsorption, on the basis of the work of Jodlbauer and Haffner (1921*b*), which indicated that the hemolytic action of non-irradiated eosine and rose bengale is dependent upon an adsorption process. He suggests (1) that the dye must be adsorbed by the cells, and (2) must retain its ability to be activated by light while in combination with the cell, thus bringing about oxidative changes. Only those dyes capable of both are photodynamically active. Those dyes which are adsorbed most readily are the most active in bringing about hemolysis in the dark, but all of these do not meet the second qualification. Thus a large number of exceptions to the general rule are accounted for. However, the hemolysis of red blood cells by previously irradiated fluorescein dyes, described by the writer (1930), renders Jodlbauer's assumption untenable, since contact of the cells and dye during irradiation is not requisite in order to produce hemolysis. The intimate contact between cells and photodynamic substance may assist in the reaction when cells and dye are irradiated together, but offers no explanation for hemolysis when the dye is separately irradiated. The hypothesis presented above offers a more satisfactory explanation of the correlation between the action of the irradiated and non-irradiated dye, in consideration of these facts.

The fact that non-irradiated photodynamic substances may produce definite changes in living cells, has been apparently disregarded by a large number of the investigators of photodynamic action. It seems, however, very important to keep this fact constantly in mind in the interpretation of photodynamic processes. This is particularly true, if we consider the effects of irradiation to be superimposed upon the effects of the non-irradiated dye as here suggested. Whatever the nature of the action of the non-irradiated eosine in producing hemolysis and fixation, we are certain that it may precipitate cell constituents, as shown by the formation of precipitates with hemolyzed cells. The fact that hemolysis and fixation are shifted in the same way by hydrogen ion concentration and by irradiation as is precipitation, suggests that the three processes are closely connected, and the possibility that the two former may be dependent upon changes in the solubility of cell constituents. At any rate, such alterations in solubility of cell constituents must be considered as a possible result of the treatment of biological substances with eosine and other photodynamic substances. We must also consider the possibility of oxidation and of changes in hydrogen ion concentration when biological substances are subjected to the action of irradiated solutions of these photodynamic agents. It is hardly possible to imagine three factors better calculated to alter cell processes than these. It seems, therefore, that until much more careful studies of the varied photodynamic phenomena are made, we need not seek farther for their explanation. It is at least unnecessary to assume unknown processes until the possibilities of the three above-mentioned clearly demonstrable factors are exhausted.

Summary

1. Hemolysis is not the only manifestation of the photodynamic effect of eosine on red blood cells, fixation and precipitation of the

hemolyzed cell constituents also occurring at proper concentrations of the dye.

2. Hemolysis, fixation, and precipitation of the cell constituents are brought about by non-irradiated eosine at proper hydrogen ion concentration and concentration of the dye. Irradiation shifts the region of occurrence of these phenomena into lower concentrations of the dye.

3. In unbuffered solutions of eosine the hydrogen ion concentration increased in the course of irradiation.

4. Hydrogen peroxide may produce hemolysis and fixation in proper concentrations.

5. Hydrogen peroxide and eosine may reënforce each other in producing hemolysis and fixation.

6. Photodynamic action may probably be regarded as a summation of the effects of the non-irradiated photodynamic substance together with the oxidative changes brought about by this substance due to irradiation.

7. Oxidative changes in cell constituents, and changes in hydrogen ion concentration, together with the change in solubility of cell constituents brought about by the photodynamic substance, may possibly account for all the phenomena generally classed under the terms *photodynamic action* or *photodynamic sensitization.*

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REACTIONS OF *CERCARIA HAMATA* TO LIGHT AND TO MECHANICAL STIMULI

HARRY M. MILLER, JR., AND ELSIE E. MAHAFFY

Zoölogical Laboratory, Washington University, St. Louis

The swimming behavior of a holostome larva, *Cercaria hamata* Miller, 1923, which McCoy (1928) found to penetrate sunfishes and develop in them to the meta-cercarial stage, has been studied, as well as the effect of light upon its activity and its reactions to shadow and to mechanical stimuli. Great variety in the swimming behavior and reactions of larval trematodes, particularly to shadowing, has been found among more than fifty species studied in more or less detail by one of us, (Miller, 1927–1930). In view of the meager knowledge of the life histories of these trematodes any statement concerning the adaptive value of the behavior of any species would be mere speculation.

In the development of the work certain facts of general physiological interest were found, and the results of preliminary studies are reported in this paper. The most outstanding is the fact that a dual mechanism of stimulation is involved in the response to mechanical stimulation and to a change of light intensity (shadow).

Material and Methods

The snail host of *C. hamata* is *Planorbis trivolvis* Say. Snails from Ramona Lake, St. Louis County, were isolated in water in shell vials; cercariæ emerged, usually in large numbers, into the water from those snails in which the infestation was mature. The snails were easily kept alive in the laboratory and were isolated from time to time to secure cercariæ. It is assumed that these cercariæ, which are fully formed and emerge spontaneously, constitute a uniform material for experimental purposes. They live approximately 48 hours after emergence, during which period they do not feed or reproduce; hence these factors do not enter into their behavior.

Observations and experiments were made in the laboratory before a window, using diffuse daylight or sunlight, and were also carried out in a darkroom. The emerged cercariæ were placed in glass spectroscope absorption cells (40 × 40 × 20 mm.) or in small cells of various sizes made from high grade glass slides and DeKhotinsky cement. All observations were made with a binocular microscope held in a horizontal position.

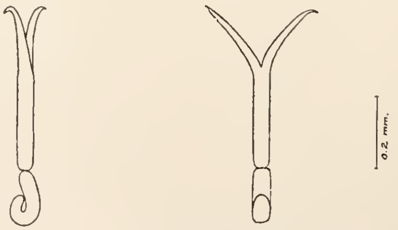
Intervals of five minutes or longer were measured with a " Reminder " clock and one minute intervals by the aid of an Eastman timer, the hand dipping into mercury and making contact every minute with a buzzer circuit. Short intervals were measured with a split-second timer registering tenths of seconds.

The light source in the darkroom was a 115 volt-200 watt, concentrated filament Mazda lamp in a 200 cm. beaver board tunnel, painted flat black. A camera shutter with a 1 inch opening was installed in the end of the tunnel. The time-setting lever was connected to an electromagnet and was operated by tapping a key. Observation in the " dark " was made by means of a weak red darkroom light behind the cell containing the cercaria. A large water cell was placed in front of the red light to absorb any heat given off.

In experiments where temperature of the water was controlled, the cell containing cercariæ was placed in a water bath and a mercury thermostat was used, connected with a heating coil and a 6-volt storage battery.

Swimming Behavior

Cercaria hamata is an intermittent swimmer. Ordinarily a short rapid upward swim alternates with a long period of sinking. The path of a swim may be changed suddenly and erratically, and occasionally an individual will make an uninterrupted swim lasting as long as 6 seconds, with many changes in direction. The tail is directed forward in locomotion, and lashes vigorously. At the end of a swim the anterior third of the body bends ventrally and posteriorly, so that the body is in the form of a hook (Text-figure), and as a cercaria sinks it slowly comes to a position with the body down. The body retains this hook-shape until swimming is suddenly resumed. At the beginning of a sinking period the forks of the tail are spread at an angle of slightly less than 180°, but this angle gradually decreases until it is about 90°.



Diagrams of lateral and ventral views of *Cercaria hamata* sinking through water.

A great deal of variation was found among individual cercariæ in the length of a swim and of the succeeding sinking period. Ten alternating periods of sinking and swimming were measured for 75 cercariæ; sometimes a long swim followed a long period of sinking, other times a short swim followed, and thus there seems to be no correlation between them. Tables I and II show the ten observations on one individual and the averages for 10 others.

TABLE I

Variation in periods of passive sinking and of alternate spontaneous swims of one cercaria.

	Period of sinking	Duration of following swim
	seconds	*seconds*
	25.2	2.2
	48.0	3.2
	34.0	2.2
	41.0	3.0
	45.0	1.8
	107.0	2.4
	20.2	1.2
	55.6	1.0
	50.4	1.2
	39.4	3.8
Average	46.6	2.2
Range	20.2–107.0	1.0–3.8

TABLE II

Averages of periods of passive sinking and of alternate spontaneous swims of ten cercariæ.

	Average period of sinking	Average duration of swim
	seconds	*seconds*
	31.0	2.4
	45.0	1.4
	42.8	0.8
	42.7	1.2
	48.6	1.3
	80.1	1.2
	39.5	1.2
	57.1	1.2
	79.0	0.9
	26.9	1.2
Range	26.9–80.1	0.8–2.4

Effects of Temperature on Activity

The temperature of the water was noted in every case; it was found that the average period of swim did not change significantly in warming the water from 18° to 35° C., but the length of the period of sinking decreased, so that swims were more frequent at the higher temperatures.

Below 15° C. swimming behavior varied from the preceding account. The body was usually held extended when the cercaria came to rest, instead of bending. In place of swimming the cercaria exhibited jerking and lashing movements; the body sometimes bent and unbent, and occasionally, held at full length, lashed back and forth. Convulsive twitching of body and tail occurred, and the forks of the tail jerked separately. When the cercaria swam, it was only for a short distance. One individual kept up the twitching movements continuously for over 21 minutes, after which it sank to the bottom. Below 7° C. the cercariæ were either motionless on the bottom or jerked about only occasionally. Above 38° C. the body of the cercaria was held extended, but twitching did not occur.

Effect of Light on Swimming

It was observed that *C. hamata* swam more frequently in strong than in weak light; this was later tested in a darkroom. Thirty groups of ten cercariæ each were used; they were placed in a small cell and dark-adapted for an initial period of 20 or 30 minutes. During the entire experiment a weak red observation light was burning. After the cercariæ were dark-adapted, the number of swims during a one minute period was counted, and then light admitted by means of the shutter and the number of swims during a one minute period in the light was counted. These procedures were repeated, with five minute periods of dark-adaptation followed by the admission of light of different intensities. In each case the number of swims in the "dark" and in the light was counted. The following approximate light intensities, obtained by placing a 115 volt-200 watt lamp at different distances from the cell, were used: 2000, 1550, 1100, 650, 200, 100, 50 meter candles. Each group of ten cercariæ was subjected only once to each light intensity; the different intensities were used in alternation of low and high intensities so that the factor of progression would not enter.

The summary of observations on the 30 groups of cercariæ is shown in Table III. The average number of swims at each intensity was compared with the average number during the preceding one minute period of dark, and the percentage of increased activity calculated.

REACTION TO SHADOWING

When *C. hamata* is in a resting state, it is usually stimulated to swim, instantaneously, either by the shadow of an opaque object or by other means of decreasing the light intensity. It was thought that when failure to swim occurred it might be due to the fact that the stimulus was applied just at, or too soon after, the cessation of a spontaneous swim. Experiments were carried out on about forty individuals to test this hypothesis. A shadow was thrown upon a cercaria at the instant that it ceased swimming, and at varying intervals (1 to 10 seconds) after the cessation. Each cercaria was allowed to swim spontaneously twice between trials. A great deal of variability was found. Some individuals almost invariably swam when shadowed as soon as they came to rest; but in general the longer the interval between cessation of swimming and stimulation, the more certain the response.

TABLE III

Effect of Light on Frequency of Swim of Cercaria hamata

Thirty groups of 10 cercariæ used. Total number of swims during one minute of dark and during a succeeding one minute period in light of a given intensity counted, and percentage of increased activity in the light calculated. Five minute intervals between trials. High and low intensities used, alternately; each group of cercariæ exposed only once to a given intensity.

Light Intensity in Meter Candles..	2000	1550	1100	650	200	100	50
Average Per cent of Increased Activity.....................	626	585	408	228	94	12	8

When a shadow is thrown upon a cell containing a large number of cercariæ, concerted, or almost concerted, swimming results. *C. hamata* is stimulated to swim by touch and by currents of water, and these stimuli, occasioned by individuals which responded to the shadow, are undoubtedly factors in the general response. When the cercariæ are isolated these factors of mechanical stimulation are eliminated.

Cercariæ of this species are inhibited by repeated shadows, after responding to the first or second of the series, when an interval of one or two seconds intervenes between shadows. After a lapse of a minute or more almost all of the cercariæ swim again when shadowed, and again may be inhibited by repeated stimuli. This may be shown by the following experiment. The number of swims made by five cercariæ during 90 seconds was counted, and then a shadow cast on the cercariæ every second and the number of swims during 90 seconds again counted, and the procedures repeated. The first shadow always brought about a response on the part of many individuals; this initial response was ignored and the number of swims in the ensuing 90 seconds counted. The data are shown in Table IV.

The effect of temperatures through a range from below 10° C. to 35° C. was studied in reference to response to shadowing; isolated cercariæ and groups of five individuals were used. Below 10° C. only an occasional cercaria swam when shadowed; from 10° C. to 35° C. the curve is practically a straight line, with 10 per cent of the cercariæ responding at 15° C. and 70 per cent at 35° C.

Table IV

Showing the inhibiting effects of repeated stimulation (shadowing) on the activity of C. hamata.

	Number of swims of 5 cercariae, counted in alternate 90 second periods							
Not stimulated	24		26		27		23	
Cercariae shadowed every second; swims in response to the first stimulus not counted		2		7		5		6

Response to Mechanical Stimulation

C. hamata when sinking quietly may be stimulated to swim instantly by mechanical stimuli, such as touch on any part of the body or tail, or jarring the container, or a fine stream of water. Whereas repeated stimulation by shadowing at short intervals resulted in failure to swim, it was found that this cercaria may be kept in almost continuous locomotion by repeated stimulation by touch. Forty cercariæ were used. Each was isolated in a small cell and touched twenty-five times with a fine wire on the body, tail-stem, or a fork of the tail.

In the case of twenty cercariæ the stimulation was applied as soon as possible after the individual had stopped swimming. Only seventeen failures to respond occurred in the total of 500 trials; twelve of the cercariæ swam every time. The other twenty cercariæ were permitted to assume a vertical sinking position before being touched; this allowed them an average recovery period of about 15 seconds. Thirty-five failures to swim occurred during 500 trials; seven of the cercariæ had a perfect response record for the twenty-five trials.

Several other individuals were repeatedly stimulated until fatigued.

One of these, allowed to resume the vertical sinking position between stimuli, responded 120 times and then failed to swim when stimulated ten times at short intervals. Another was touched " immediately " upon cessation of each swim. It responded to 79 successive stimuli, but after the fiftieth trial the swims became shorter and near the end of the trials the reaction consisted only of a few vibrations.

From these data and those on response to shadow stimuli, it seems clear that two different mechanisms are involved; a relatively long interval is necessary to secure anything like a regular response to the shadow stimulus, when repeated, whereas a very brief interval is sufficient in the case of stimulation by touch.

Effect of Temperature.—Between 20° C. and 38° C., the cercariæ usually swam when touched by a needle, but at lower temperatures a high percentage of failure occurred. Below 10° C. the response consisted of the jerkings which are the only movements found at these temperatures.

TABLE V

Comparison of duration of spontaneous swim with that following stimulation by shadow, touch, and stream of water. Duration of swimming in seconds.

	Cercaria *a*	Cercaria *b*
Unstimulated	1.16 ± 0.17	0.69 ± 0.07
Stimulation by:		
Shadow	2.15 ± 0.16	1.84 ± 0.17
Touch	3.24 ± 0.29	1.95 ± 0.16
Stream of Water	2.66 ± 0.24	2.01 ± 0.16

COMPARISON OF SPONTANEOUS WITH STIMULATED SWIMMING

It was observed that when *C. hamata* swam spontaneously, the average duration of swim was shorter than when it was stimulated by any of the methods used. This was tested experimentally on a number of individual cercariæ, in the following manner. The duration of a spontaneous swim was measured, and then fifteen seconds after the cercaria had stopped a shadow was cast upon it and the duration of the resulting swim measured. This was followed by touch with a fine wire and stimulation by a fine stream of water from a pipette, and the series was repeated twenty-five times for each individual. The cercaria was always allowed to swim twice between stimuli and the stimulus was then applied 15 seconds after the cessation of the second swim; occasionally the duration of the period of sinking was shorter than 15 seconds, in which event three spontaneous swims intervened between stimuli.

Data for one hundred observations on each of two cercariæ are given in Table V. It was calculated that the difference between the mean for the spontaneous swims and those for the three types of stimuli is 4.2 (shadow), 6.2 (touch), and 5.1 (stream of water) times the probable errors in the case of cercaria *a*, and 6.2 (shadow), 7.2 (touch), and 7.4 (stream of water) in the case of cercaria *b*. For nine other cercariæ the probable errors were not calculated, but it is evident that the differences are statistically significant.

Discussion

Reactions of the sort described in this paper have been found in some species of most of the large groups of animals, but among the larval trematodes they are quite common, particularly the response to shadowing. The extent to which these varied responses aid cercariæ to reach an intermediate or final host has been studied in only one instance. Miller and McCoy (1929; and in press) have shown that the reaction of *Cercaria floridensis* to shadows, cast by fish intermediate hosts, carried the cercaria to the upper levels of the water and hence possibly was a factor in the infestation of the fishes. On the other hand, Cort and Brooks (1928), who studied the characteristic behavior but not the reactions to stimuli, of a number of fish-penetrating holostome larvæ, believe that the activity of the fish intermediate hosts is such that they would come in contact with the cercariæ, so that there is no necessity for activity on the part of the latter. Until experiments have been carried out on each species, the adaptive value of these reactions to shadow and to mechanical stimuli cannot be determined. The reactions to shadow described for *C. hamata* could be brought about in nature by animals swimming above the cercariæ, and the swimming upward would carry the cercaria toward a possible host. Judging from Cort's studies (1922) on the escape of cercariæ from their snail hosts it is likely that *Cercaria hamata* would emerge in but small numbers at temperatures below 17° C., and, on the basis of the present data on effect of low temperatures, would only occasionally react to a shadow cast by a possible host.

From the results of the present preliminary study it seems evident that a dual mechanism is involved in the response to shadow and to touch, because the cercaria may be kept in almost continuous locomotion by touch stimuli, whereas a relatively long interval must intervene between shadow stimuli to secure anything like a regular response. Experiments are now under way which are so planned as to elucidate further the nature of this dual mechanism. Effects of curare, nicotine, and other substances which specifically affect myoneural junctions are being studied, as well as the effects of polarizing currents, ultra-sonic radiation, and other forms of stimulation.

SUMMARY

1. *Cercaria hamata* swims intermittently. A short, rapid swim alternates with a relatively long period of quiet sinking.

2. The duration of the period of sinking decreases with rise in temperature. Below 15° C. the cercariæ exhibit jerking and lashing movements in place of swimming.

3. Shadowing a dense group of cercariæ results instantly in the almost simultaneous swimming of most of them. The response is partly due to the shadow stimulus and partly to the mechanical stimulus occasioned by the colliding of active individuals with quiet ones.

4. Repeated shadowing at short intervals results in inhibition of swimming.

5. Mechanical stimulation by touch (or a stream of water) initiates swimming instantaneously. Only a very short interval between stimuli is necessary to keep a cercaria in almost continuous locomotion. This indicates that a dual mechanism is involved in response to shadowing and to touch.

6. The duration of swim in response to shadow, touch, and stimulation by a stream of water is significantly greater than that of a spontaneous swim.

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ON TEMPERATURE AND THE BREATHING RHYTHM OF *CANIS MUSTELUS* AND *SQUALUS ACANTHIAS*

A. E. NAVEZ

(From the Laboratory of General Physiology, Harvard University, Cambridge, Massachusetts)

The casual observation of dogfishes in aquaria reveals immediately a noteworthy regularity in the rhythmic movement of the gill-slits. The movements of the spiracles are coördinated in a perfect way with those of the slits and they go on with the same regularity, without interruption, for long periods. All these features seemed to point to the suitability of this animal for a study of the frequency of gill movements as controlled by temperature.

The previous work of Bethe, Baglioni, and others had shown considerable irregularity in the Q_{10} ratio (*cf.* Kanitz, 1915); the observations of Crozier and Stier (1924–25) on the opercular breathing rhythm in the goldfish, on the contrary, showed regularity in this rhythm under properly controlled conditions and demonstrated the applicability of the Arrhenius equation to the data obtained. Would the dogfish obey the same rule and in a similar way?

For the observations, young specimens (25 to 37 cm. long) were kept in a large aquarium the temperature of which was maintained constant to within ± 0.05° C. for periods of 30 to 50 minutes at any given temperature. The number of points on the thermometric scale chosen for the observations was made as large as possible, and in general at each temperature from 20 to 30 series of 20 beats were timed with a stop watch. The regularity of the breathing rhythm at constant temperature is remarkable; countings of 20 to 30 successive series of 20 beats often agreed to within 1 to 2 per cent. The only exception seems to be around 15° C. where more erratic values are found. We will come back later to this point. Let us note also that Crozier and Stier have found agreement of the same order of magnitude. Care was taken to allow time for complete temperature equilibrium between the organism and the medium, the water of the aquarium also being stirred gently. The aquarium was almost completely insulated by a cover which cut out most of the light; a small portion of the surface only was left uncovered to permit observation. The water in the aquarium was kept as close as possible to saturation with air, so as to exclude any possible effect of O_2 concentration. Observations made with waters having different concentrations of O_2 did not show any direct effect of oxygen concentration upon frequency of breathing movements, at least in the range considered. The first observations showed immediately the importance of having the animals perfectly quiet. Measurements can only be made if the animals have not been actively swimming for at least five minutes, the gill rhythm being speeded up after swimming. This is in harmony with the observations of Parker (1909) ". . . that the rate of gill movement in the dogfish depends upon the momentary state of movement of the animal. When resting they vary from 35 to 40 movements per minute. When swimming slowly they respire 50 to 55 times per minute. In vigorous swimming, the rate is doubtless still more rapid." These observations were made probably between 15° and 18° C., and the frequencies agree fairly well with those here found.

The first indications given by the observations show that the values for Q_{10} are rather erratic, as will be seen in Table I.

TABLE I

Q_{10} ratios

$\frac{11.4° \text{ C.}}{21.5° \text{ C.}} = 2.06$	$\frac{12.70° \text{ C.}}{22.75° \text{ C.}} = 2.38$	$\frac{13.5° \text{ C.}}{23.5° \text{ C.}} = 1.72$
$\frac{14.5° \text{ C.}}{24.5° \text{ C.}} = 1.78$	$\frac{15.5° \text{ C.}}{20.5° \text{ C.}} = 1.21$	$\frac{17.5° \text{ C.}}{22.75° \text{ C.}} = 2.10$

The data were treated to determine the goodness of fit of the Arrhenius equation (Fig. 1). The graph shows immediately that nearly all the points are distributed within three main bands, each with parallel edges; a fourth, small, short band parallel to one of the three main ones, contains the rest of the points. These bands may be called *I, Ia; II, III* (*cf.* Fig. 1). The values found for μ are:

I and *Ia*	16,400
II	8,200
III	35,000

The first of these values corresponds exactly with that found for the opercular breathing rhythm of the goldfish by Crozier and Stier (1924–25). But, differing from what was found in the latter case, where only one temperature characteristic was secured, two additional values of μ appear (8,200 and 35,000). One should note the fact that the observational points which fall in *Ia,* were followed suddenly by points falling in the upper part of band *II,* without any smooth transition. The absolute values for the points in *Ia* are very different from those in *I,* but the slopes of both *I* and *Ia* are identical.

Another shift of points occurs also for *I*, where under certain not well-determined circumstances, in the neighborhood of 14.5° C., a sudden shift to *II* may be found. These cases of shift from one band to another are analogous to the ones found by Crozier and Stier (1925–26, p. 552) with the goldfish, after the animal was kept for 3 hours at 25° C. They agree with records obtained in many other instances, and are therefore an excellent argument in favor of the idea of different reactions playing definite rôles within definite limits of temperature. Occasionally one finds some observational point departing totally from the rest of the collection, and in many cases these data fall exactly in

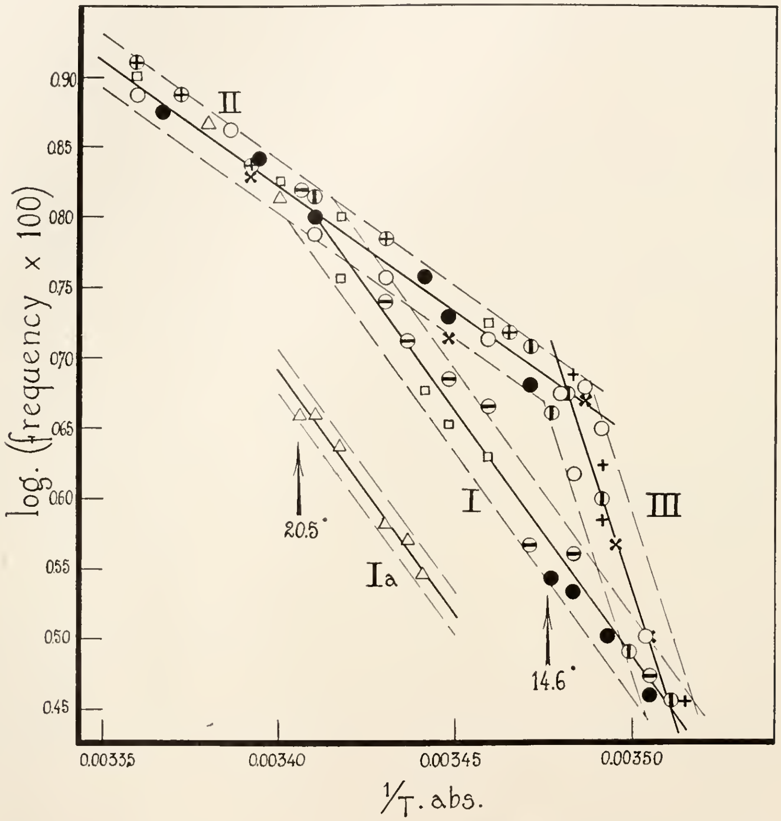

FIG. 1. Mass plot of all observations on *Mustelus canis*. Each animal has been represented by a different symbol. Each plotted point corresponds to the mean of 10 or 20 sets of readings, each reading being the time required for 20 gill movements. The number of sets of readings has been kept consistently 10 (or 20) for each animal; in other words, for any one animal there are 10 (or 20) sets of readings at each temperature.

the other band. These abrupt departures are especially frequent around critical temperatures, making these points very obvious in the graph when all observations are plotted separately. Necessarily all illegitimate averaging smooths out such points, which at the same time often lowers the absolute value of these average frequencies.

Changes of slopes of this nature are not surprising, as they have been found previously. They may perhaps indicate here that the " fundamental mechanism " determining the frequency of gill movement, which yields $\mu = 16,400$, may be in its turn linked with processes having lower (8,200) and higher (35,000) values of the critical increments. It has been suggested by Crozier and Federighi (1924–25) that the value $\mu = 8,200$ may be perhaps understood as reflecting the velocity of central nervous processes concerned in respiratory movements of fishes and other vertebrates. In all cases we may consider that the " fundamental mechanism " is by no means a simple one but must be probably of catenary type, more or less complicated, but where one of the three reactions (here characterized by their respective values of μ) plays the rôle of master reaction at any one moment.

We have not tried to find out any possible correlation existing between breathing and cardiac rhythms, as this would have obliged us to introduce in the animal pieces of apparatus which undoubtedly would have altered the breathing rhythm at least. We had noticed that the placing of a small glass rod (which has to be applied with utmost care in order to avoid all indirect stimulation) in the immediate vicinity of the opening of the spiracles affected singularly the coördination existing between spiracles and gill slits. Occasionally " beats " of the spiracles (in certain cases 3–4–5 in succession) are skipped although the gills operate at their normal constant rhythm. Scott (1913) had noticed also the relative independence of heart and gill movements, where he (p. 61) notes that the heart can continue to beat long after respiration has ceased, and that even when both organs are in action their rates may be increasing and decreasing independently of one another. On the other hand, Lyon (1926, p. 282) says that " even without artificial respiration, the heart may continue active long after breathing ceases " and on page 283 " that the heart rate of the sand shark is intimately related to the respiration rate. Usually the two are equal . . . the heart . . . normally takes its rate from respiration." One must remember that these experiments were performed with the sand shark (*Carcharias*), which has a slightly different type of water movement path than *Mustelus canis*, and that furthermore, as these animals were operated upon to introduce canulæ and connecting solutions for blood pressure determinations, and were kept partly out of water, they were far from being in a state of " no interference " to which we wished to restrict ourselves.

The respective positions of the bands in Fig. 1 determine at least two critical temperatures: *I* and *II* intersect at about 20.5° C.; *II* and *III*, at about 14.5° C. The fact that these temperatures occur as critical temperatures in other respiratory activities (*cf.*, *e.g.*, respiration of *Molge, Rana, Bufo* for 15° C., Crozier, 1924, p. 198; respiration of *Vicia faba* for 20.5° C., Navez, 1929, p. 656) is rather striking. Crozier and Stier had noticed also that at temperatures above about 15° C., the pectoral fins of the goldfish were rather active, affecting by their movements the breathing rhythm, and that below this temperature no such disturbing activity was exhibited. We pointed out previously that the latitude of variation of the data was rather small, of the order of 1 to 2 per cent. Only in the vicinity of 14.5° C. do we get a variation which is, at its maximum, 7 per cent, but which, on both sides of this temperature, reduces to its normal size. The fact that we did not get such changes in the latitude of variation for the other critical temperature seems to indicate that this variation is in itself indicative of some definite change taking place in the organism, conceivably—for example—modifications in the reactions controlling discharge in the synapses. This would be consistent with the idea of assigning the value $\mu = 8{,}000 \pm$ to processes of central nervous origin determining breathing movements in the dogfish.

Another point may be made in relation to this matter of variation. When we speak here of " variation," we mean the tendency of such data to be scattered, in a seemingly random manner, in the band (Fig. 1) that we fit to them, and do not have reference to the error or mistake that the observer could have introduced involuntarily. The variation found in the data is *in* the material, truly a property of the organism, and not due to some lack of precision in the measurements. How could we in fact understand that the observer could make such errors that would cause all his data, after averaging, taking of reciprocals and logarithms, to fall in a band with parallel edges? A variation presenting such properties ought to permit us to analyze it as a function in itself. In other words we can consider, in the behavior of the organism, the behavior of a mathematical function and try to correlate one with the other. Analogous cases have been described by Crozier (1929).

First, why are the bands with parallel edges? It might be objected that it is impossible to conceive a regularity in the observations that would give such bands. It may be urged that viscosity, and the general complexity of protoplasm, would make the scatter of observational data rather irregular. One may point out that it would be astonishing if in the very large number of cases now on record, such things as viscosity would have behaved consistently in a way that paralleled the behavior of the process considered; in other words, that viscosity and other properties should have a series of temperature characteristics always the same as that of the process under study. Furthermore, would it not be strange that from worms up to mammals the rule governing viscosity, for instance, would be similar to the one controlling respiration or cardiac rhythm? Is it not rather a case where the very inconsistent appearance of the data of some authors is indicative, more of the ways of operation of the observer than of some fundamental character so easily ascribed to protoplasm? One *could* advance several reasons why divergences from a band with parallel edges "should" be found, but in the absence of such effects they need not be considered.

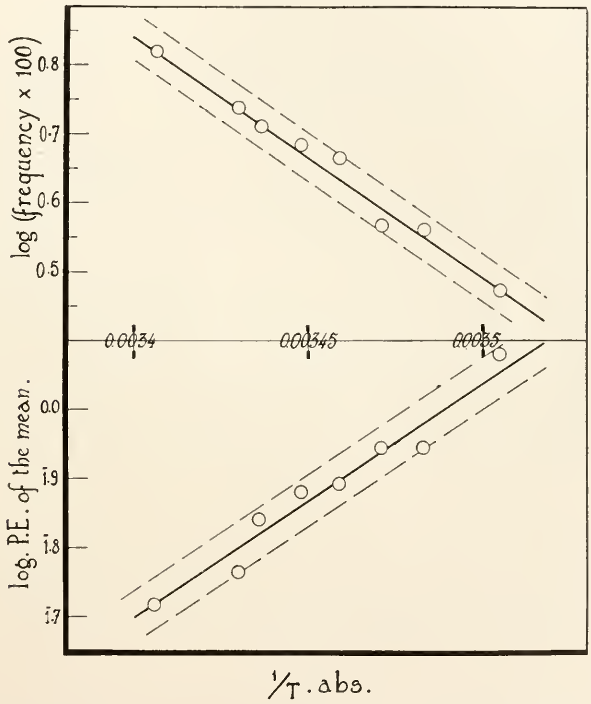

FIG. 2. The upper half of the graph is a reproduction of the points obtained for the animal represented by circles with horizontal diameters in Fig. 1. The lower half is a plot of the log of the P.E. of the mean frequencies of gill movements as a function of temperature. The slopes of the two lines are equal.

Let us go back to Fig. 1 and select out of the series of observations two sets to illustrate another point: a series (represented by a circle with an horizontal diameter) occurring in band *I*, and a second series (indicated by solid circles) occurring in bands *I* and *II*. Each one of these series, plotted separately as in Figs. 2 and 3, shows this distribution of points in bands with parallel edges. We have represented also in the lower half of each figure the relation of the P.E. of the mean time for 20 beats to temperature for each of the points represented in the upper part. In each case the slope of the best-fitting line in the P.E. graph is the same as that obtained for the mean frequencies (*cf.* Crozier, 1929). In other words, the latitude of variation of the observations is a constant fraction of the mean, and both mean and variation of the mean are affected by temperature in the same way. Their temperature characteristics are the same. This is particularly illustrated in the second case where a definite shift from one value of

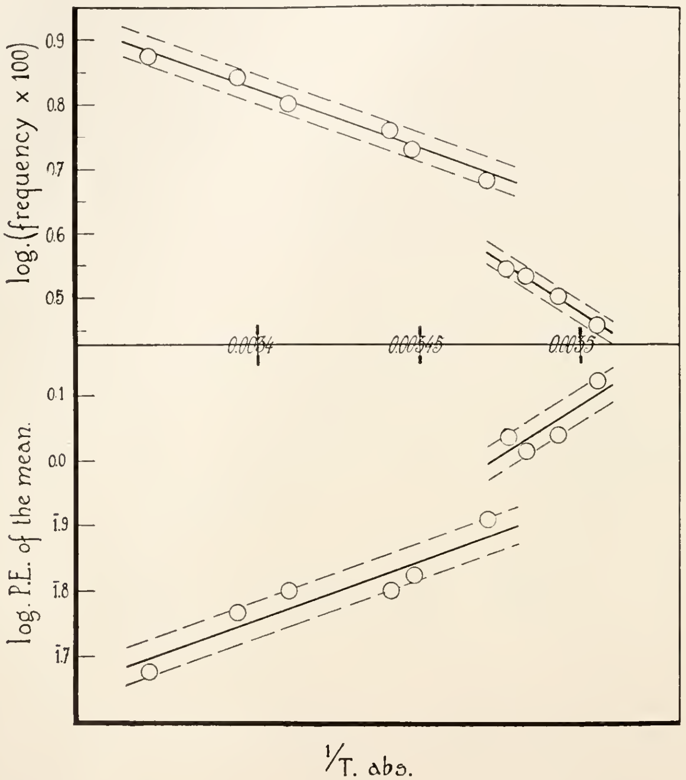

FIG. 3. The upper half of the figures is a reproduction of the points obtained for the animal represented by solid black circles in Fig. 1. The lower half is a plot of the log of the P.E. of the mean frequencies of gill movements as a function of temperature. The slopes of the lines are equal, and the break in each curve is located at the same temperature.

μ to another occurs. The P.E. curve shows the same break at the same point on the thermometric scale.

Two other conclusions can be drawn also: (1) a straight line relationship must exist between the P.E. of the mean (or the standard deviation of the mean) and the mean itself, (2) the standard deviation of the mean divided by the mean (*i.e.*, the coefficient of variability of the mean) must have no temperature characteristic (*i.e.*, the slope against 1/T must be zero. Each one of these conclusions has been illustrated in Figs. 4 and 5. [The use of σ_m (the standard deviation of the mean) instead of the P.E. affects only the size of the unit and not the form of relation.] Obviously the straight line relationship is obeyed and the seemingly best-fitting line we draw through the scattered points of Fig. 5 is a line with a slope equal to zero or very close to it.

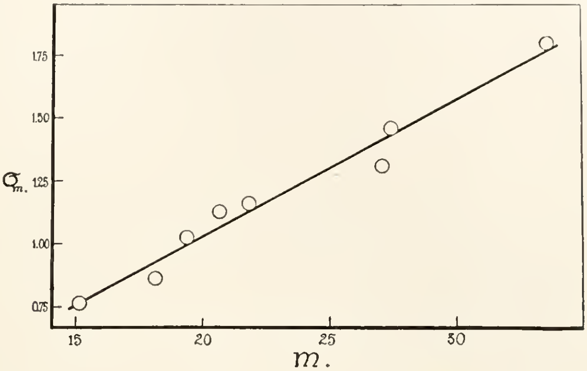

FIG. 4. The standard deviation of the mean frequency of gill movements is a linear function of the mean.

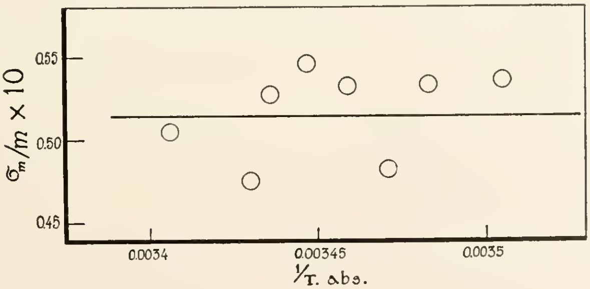

FIG. 5. The "variability" of the mean (*i.e.*, the standard deviation of the mean divided by the mean) as a function of temperature; this "variability" is constant.

An analogous case has been described by Crozier (1929, p. 95) in relation to the behavior of guinea pigs on an inclined plane. The angles of orientation assumed by the animals on the plane for different inclinations of the plane show a very marked change in their variation at a given point (45°). The probable error plotted versus the angle of tilt of the plane shows a marked break at 45°, much more marked indeed than the one in the regular plot of orientation versus tilt of plane. This change in a mathematical function can, here also, be definitely correlated with reactions in the animal: the type of creeping is different above and below 45°.

These observations lead to the conclusion that in the cases where experiments have been made in a controlled way, where averaging of data has been done with discrimination, and where, as a whole, the observations are correspondingly weighted, we can study not only the phenomenon itself, but also the laws of variation of this phenomenon. These provide an additional check on the existence of critical temperatures and enable us to fix them in a quite independent way. In such cases, the study of the P.E. and of the coefficient of variability will confirm for us the idea that biological material may not be so " variable " as it is often taken to be.

Observations were made also on the spiny dogfish (*Squalus acanthias*). These data are identical with those in Fig. 1 giving the μ value of 16,400. Here also, in a few instances we found sudden shifts to the value 35,000. As the observations were not numerous enough, they are not given in detail.

We seem to have here a case homologous with the one found by Crozier (1924) in the data of Miss Leitch (1916) on the rate of development of the radicle of *Pisum*. Crozier showed (*cf.* 1924, Fig. 13, p. 207) that in this case these increments can be found: one of 16,450 when the observations were taken over periods of 0.5 hour; a second and a third one (over different temperature ranges) when the determinations were carried over periods of 22.5 hours. This last series of increments was obtained for data which included thus the hours of darkness and the daily rhythm of cell division in the plants. The former, on the contrary, were taken over short periods which could thus " average " out any possible trend due to the moment of observation with respect to the normal division rhythm.

The possible coexistence of such a series points to the fact that any one of two (or three) factors can act at any moment as " pace maker," impressing thus its temperature characteristic on the whole system. Our case is homologous in that we also have found for different animals the shift of one increment to another indicating a corresponding shift in the pace makers of the system. Any one of these could come into control and last for a certain time, and then be replaced by another one determining another thermal increment for the system.

SUMMARY

The temperature characteristic or critical thermal increments (μ) for the movement of the spiracles and gill slits in the dogfish *Mustelus canis* (and *Squalus acanthias*) are 8,200; 16,400 and 35,000 calories. Critical temperatures are found at 14.5° C. and at 20.5° C. The latitude of variation of the measurements varies in a definite way and this is correlated with the occurrence of a critical temperature of 14.5° C.

The reactions controlling breathing movements seem to be catenary processes, one of which may be concerned with the central nervous control of the breathing movements, over a certain range of temperatures.

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HIBERNATION OF THE THIRTEEN-LINED GROUND SQUIRREL CITELLUS TRIDECEMLINEATUS (MITCHILL)

V. Food, Light, Confined Air, Precooling, Castration and Fatness in Relation to Production of Hibernation[1]

GEORGE EDWIN JOHNSON

Kansas State Agricultural College

Introduction

The cause of hibernation has been considered by many investigators and to account for it there has been presented a large number of theories, some based upon extended observations, others founded upon more or less casual studies of a small number of animals. In few instances have carefully controlled experiments been conducted. Most of these theories have been brought together by Rasmussen (1916).

Several authors admit that starvation tends to produce hibernation (Mangili, 1807; Hall, 1832; Merzbacher, 1904; Simpson, 1912; Mann, 1916; Shaw, 1925; and others). Most of these also state that little food was eaten shortly before a mammal goes into hibernation, indicating that hibernation took place when food was present and that starvation was not a factor. Dry food is considered a cause of æstivation (which is apparently a condition of lowered metabolism accompanied by a drop in body temperature, as in hibernation, but of a less degree and occurring in the summer or fall instead of in the winter) by Shaw (1925), Kashkarov and Lein (1927) and Kalaboukhov (1929).

Vitiated air was not considered as having any effect on hibernation in marmots by Mangili (1807), but Bert (1868, 1870) was able to produce lethargy in dormice by placing them at a low temperature in a bell jar deprived of air.

There appears to be an agreement among those authors who have mentioned adiposity that this condition is common in the fall among hibernators and that such a condition is favorable to hibernation, *e.g.*,

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[1] Contribution 118 from the Department of Zoölogy, Kansas State Agricultural Experiment Station, Manhattan, Kansas. Earlier papers in this series deal with conditions in hibernation (Johnson, 1928), waking from hibernation (Johnson, 1929*a*, 1929*b*), and the influence of thyroxin, pituitrin and dessiccated thymus and thyroid on hibernation (Johnson and Hanawalt, 1930). For abstracts referring to portions of this work see Anat. Rec. 31: 337 and 37: 225.

Mangili (1807), Horvath (1881), Forel (1887*a*, 1887*b*), Mills (1892, 1893), Claparède (1905), and Mann (1916). According to Rasmussen (1916), this view was also presented by Sacc (1858).

No study has apparently been made relative to the effects of light and darkness, precooling, or castration, although Mann (1916) usually placed his ground squirrels in the dark in addition to starving them and cooling the room when he wished to produce hibernation.

The present work was begun in 1925 and has been continued to 1930 as time and numbers of animals available have permitted. Ground squirrels of the variety *Citellus tridecemlineatus pallidus* Allen from western Kansas were used. Since this variety has been broken up by Howell (Proc. Biol. Soc. Wash., 41: 211), it would seem that some at least of the animals used would belong to the southern group (*C. t. arenicola*) of what has previously been taken as all belonging to the variety or subspecies *C. t. pallidus*. Large numbers of ground squirrels could usually be obtained, so that the data of the different experiments could be treated statistically, thereby eliminating, to a large extent, personal judgment. Furthermore, for each animal placed under experimental conditions, another of like weight and sex was usually placed under conditions that were identical except for the one factor being tested, *e.g.*, food, confined air, etc. Observations soon showed that there was considerable individual variation and that the use of large numbers was necessary in order to make the results reliable. The work was done partly in a refrigeration room in which there was some daily fluctuation of temperature, but chiefly in an automatically controlled electric refrigerator in which there was very little fluctuation of temperature.

EXPERIMENTAL

Starvation and Darkness

These two conditions were studied together in nineteen experiments, from January to October, 1925. Each experiment lasted between 7 and 23 days. Three ground squirrels were fed in the light, three were fed in the dark, three were starved in the light, and three were starved in the dark. The starved animals were given water, green feed and, in most of the experiments, five kernels of corn daily. The fed animals were given water, green feed and liberal quantities of oats, with some wheat or corn at times. The light which was used to illuminate half of the cages was furnished by one 100-watt and one 50-watt electric bulb which were kept going throughout the experiments. The lights were at a distance of about six feet from the cages. The temperature of the cold room, which was approximately a ten-foot cube, showed an average range of daily fluctuation of 6° C.

At the end of an experiment the 12 animals were removed to the heated animal house and fed daily. Usually these were returned to the cold room in a new experiment in about two weeks and given treatment opposite to that in their previous period to balance out individual tendencies if there were any. It also avoided starving any animal at close intervals.

TABLE I

Food and light in relation to hibernation. Summary of daily records in the cold room.

The total days that the three animals were in the refrigeration room are given within the parentheses; the total days before hibernation for each group of three are given before the parentheses; the latter divided by the former gives the percentage of days before hibernation for each group of three animals and is given in the per cent column.

Date begun 1925	Time in days	Fed and light		Fed and dark		Starved and light		Starved and dark	
		Total days	Per cent	Total days	Per cent	Total days	Per cent	Total days	Per cent
Jan. 6	7	20 (21)	95	6 (21)	29	10 (21)	48	0 (21)	0
Jan. 14	9	16 (24)	67	9 (25)	36	5 (22)	23	5 (25)	20
Jan. 24	11	2 (33)	61	17 (33)	52	8 (33)	24	15 (33)	45
Feb. 4	12	14 (36)	39	25 (33)	76	7 (36)	19	5 (33)	15
Feb. 17	13	39 (39)	100	33 (39)	85	12 (39)	31	24 (39)	62
Mar. 3	14	42 (42)	100	42 (42)	100	11 (42)	21	12 (42)	29
Mar. 19	14	42 (42)	100	29 (39)	74	10 (42)	24	24 (26)	92
Apr. 2	16	48 (48)	100	42 (42)	100	7 (48)	15	6 (14)	43
May 7	23	56 (69)	81	69 (69)	100	34 (66)	52	66 (66)	100
May 30	15	45 (45)	100	45 (45)	100	23 (45)	51	7 (45)	16
June 16	18	54 (54)	100	43 (54)	80	31 (54)	57	30 (54)	56
July 4	13	39 (39)	100	37 (39)	95	26 (39)	67	24 (39)	62
July 18	13	26 (26)	100	39 (39)	100	10 (26)	38	29 (39)	74
Aug. 2	13	39 (39)	100	39 (39)	100	15 (39)	38	15 (39)	38
Aug. 15	13	27 (39)	69	27 (39)	69	3 (39)	8	11 (39)	28
Aug. 30	12	24 (24)	100	18 (36)	50	6 (24)	25	7 (24)	29
Sept. 11	11	7 (33)	21	15 (33)	45	23 (33)	70	14 (33)	42
Sept. 22	15	33 (45)	73	30 (45)	67	26 (45)	58	16 (24)	67
Oct. 8	16	34 (48)	71	9 (48)	19	3 (48)	6	17 (48)	35
Total days		607 (746)		574 (760)		270 (741)		327 (683)	
Average percentages		81.4		75.5		36.2		47.8	

In tabulating and evaluating the results (Table I) the number of animals given the same treatment (*e.g.*, given feed in the light) is multiplied by the days duration of the experiment (7 in the first experiment, to 23 in the ninth) to obtain the total days in the cold room, given in parentheses. The days spent in the cold room by each of the three animals before going into hibernation are added to obtain the total days before hibernation, placed before the parentheses. The latter divided by the former gives the percentage of days before hibernation. The totals of the experiments are brought together in Table II and the percentage of days before hibernation computed for each of the four conditions, fed and light, fed and dark, starved and light, and starved and dark. When the percentages of days before hibernation are compared, it is observed that both of the fed groups averaged much higher than the starved groups. Feeding therefore delays hibernation, or starvation hastens entrance into this condition. The two "dark" groups show no marked difference from their corresponding "light" groups. Light or darkness appears to play very little, if any, part, then, in inducing hibernation.

TABLE II

Summary of Percentages of Days Before Hibernation from Table I

	Fed vs.	Starved
Light	81.4	36.2
Dark	75.5	47.8
Averages	78.5	42.0

	Light vs.	Dark
Fed	81.4	75.5
Starved	36.2	47.8
Averages	58.8	61.6

Summary of Percentages of Days in Hibernation from a Table Not Published

	Fed vs.	Starved
Light	10.9	48.0
Dark	15.2	36.8
Averages	13.0	42.4

	Light vs.	Dark
Fed	10.9	15.2
Starved	48.0	36.8
Averages	29.4	26.0

The data have similarly been tabulated to show the percentage of days in hibernation as a different measure of the production of hibernation. Only the summary of this table is included in Table II. The average percentage of days in hibernation is much higher for the starved groups than for the corresponding fed groups, showing in a different way the effectiveness of starvation in the production of hibernation. Here also there is no marked difference between the two dark and the two light groups. The difference is still less when the average of the two light groups is compared with that of the two dark groups. Statistical treatment showed that the results between each fed group and its corresponding control was significant (Difference/Probable Error $=$ 11.3 in one case and 6.3 in the other case). The difference between the "light" and "dark" groups was not significant (D/PE = 1.6 and 2.4, respectively).

It appears safe to conclude from these experiments that starvation is a condition favorable to the production of hibernation, whereas darkness is no more conducive to hibernation than artificial light of a strength such as is used in an ordinary well-lighted room. Whether ultra-violet light might stimulate the animals to stay awake has not been investigated.

Confined Air

Preliminary experiments in which some animals had been placed in half-gallon tin cans with tight lids in which were punched only two six-penny nail holes about 3.0 mm. in diameter resulted in death in some of the animals. Four such holes, however, appeared to admit sufficient air to prevent suffocation at refrigerator temperatures. In each experiment three animals were placed in the four-hole cans, three were placed in open wire cages (6 x 6 x 10 inches) as controls and three others were placed in half-gallon cans with highly perforated lids to admit an abundance of air. The latter controls were used to determine the effect, if any, of the limited space on the experimental (confined air) animals. Except in a few short preliminary experiments the animals were fed whole oats, wheat or corn, and a small amount of green grass or sprouted oats daily. Wood shavings were used as nest material and in the cans also served to absorb the moisture from the breath of the animal. Each experiment was usually terminated after 7 days. The animals were usually undisturbed except for the daily observations. All the animals were in the dark.

From Table III it is to be noted that in all four sets of experiments or periods the confined air animals went into hibernation sooner and stayed in more of the time than the animals in the open cages. A summary of the results of these experiments shows that those animals in confined air went into hibernation after 19 per cent of the total days they were in the refrigerator as compared to 49 per cent in the case of the open cage animals. These results are quite significant (D/PE = 9.5) in showing the retarding effect of free air upon entrance into hibernation. The confined air animals were in hibernation 68 per cent of the days in the refrigerator, whereas those in open cages were in hibernation only 41 per cent of the days. These results are also significant (D/PE = 9.4). The total differences of periods 1 and 2 (Table III) and also of periods 1, 2 and 3 were found to be significant.

The confined air animals also showed an earlier entrance into hibernation and more days in hibernation than did those in the cans with

TABLE III

Results of Confined Air Experiments

Period	Experimentals in Cans with four holes			Controls in cans with many holes			Controls in open wire cages		
	No. of Animals	Per cent of Days		No. of Animals	Per cent of Days		No. of Animals	Per cent of Days	
		Before hibernation	*In* hibernation		*Before* hibernation	*In* hibernation		*Before* hibernation	*In* hibernation
April 1925 to June 1925	18	15	74	—	—	—	16	81	15
Nov. 1925 to June 1927	50	20	66	51	33	46	51	39	49
June 1927 to Oct. 1927	27	14	73	25	55	37	25	52	38
Sept. 1929 to Feb. 1930	13	26	59	15	31	56	15	42	48
Total Period* April 1925 to Feb. 1930	108	19	68				108	49	41
Period* Nov. 1925 to Feb. 1930	90	19	67	91	39	45	91	43	45

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* Owing to the difference in number of animals in the groups above, these percentages are not averaged from the above averages, but from a table which contains the percentages for each animal.

many holes. The percentage of days before hibernation was 19 in the case of the experimental and 39 in the case of the controls in the many-hole cans (D/PE = 5). The percentage of days in hibernation was 67 for the experimentals and only 45 for the controls in many-hole cans (D/PE = 6.7). These results show a significant increase in early entrance into hibernation and continuance in this state because of the confined air, and show that the limited space of the cans played little, if any, part in the production of hibernation in the four-hole cans. It is to be noted, however, that in the last experiments (13–15 animals) nearly as much hibernation occurred in the cans with many holes as in those with four holes. In the other groups of experiments which included larger numbers of animals this was not the case. In these last experiments two conditions were unfavorable to hibernation in the four-hole cans. One was that the refrigerator was running very little and did not freeze the condensed moisture in the refrigerator as in active refrigeration, resulting in a high humidity in the cans. The other was that the animals were placed in the four-hole cans just as soon as placed in the refrigerator. Furthermore, the temperature in the refrigerator was not as low as usual. In this connection it should be stated that one condition tending to retard hibernation in the four-hole cans, in most of the experiments, was the retention of heat in these cans as long as the animal was active, *i.e.,* not torpid. Observations showed the temperature to be higher in these cans than in the cans with many holes. In some cases the animal appeared to be irritated by the confined air if it did not soon become torpid, and in the last series of experiments some of the confined air animals died.

Two methods of lowering the temperature in the four-hole cans were tried. In one experiment these cans were placed on a shelf below the others which was not over one degree cooler. In three other experiments the four-hole cans were wrapped on the sides by a small towel and then set in a shallow pan of water. Evaporation tended to cool the can one to three degrees below the temperature of the cans with many holes when the animals were normal in both. To avoid overcooling the four-hole cans, it was deemed best to place them on the same shelf without additional cooling by wet towels, etc., and permit the higher temperature to be a handicap to hibernation. Confined air, therefore, is probably more effective in the production of hibernation than indicated by these experiments and doubtless aids production of hibernation in nature, since the animals plug the entrance into their burrows when they retire in the fall. The limited space played very little part in the production of hibernation, possibly because the animals usually roll up in the nest whether in a large or small cage except when eating. Most of these animals did not actively try to escape from the cages or cans.

Precooling

Proponents of the view that hibernation is produced not by external conditions, but by an internal seasonal rhythm, have pointed to the greater difficulty in producing hibernation in the summer with artificial cold than in winter. While less tendency to hibernate in the spring and early summer than in the early winter has been noted by the author, it must be recognized that several external conditions may be different in the fall from those obtaining in the spring and summer. The effects of a previous season of hibernation, the poor condition of the wild animal when caught for early summer study, and the greater excitability of new wild animals also usually caught just before the summer experiments are in contrast with the conditions produced by keeping the animals one to several months for the study of winter hibernation. The observations made so far suggest that these conditions influence hibernation. In the summer, also, animals are likely to be removed directly from a warm room into a refrigerator, whereas in the fall they are often taken from a room which has a low and possibly fluctuating temperature. This precooling of the animal nightly in an animal house is almost unavoidable unless special pains are taken to control the temperature. Drops in room temperature to 60° or even 40° C. would not be unusual under ordinary conditions of heating.

The effect of this condition of daily precooling has been investigated in seven experiments. In each experiment 10 animals were taken to a cold cave or attic where the temperature averaged about 16° or 17° C. for an average of about eight hours in one experiment and 15 hours in the others for a period of about ten days. Ten controls were kept in the animal house at an average temperature of about 26° C. or about 80° F. The animals in the cave or attic occasionally became partly torpid, but controls in the animal house did not hibernate there.

After this preliminary treatment, the animals were all removed to a refrigerator kept at a temperature which was constant or changed very slowly. The average temperature was about 7 or 8° C. Experimental (precooled) and control animals were on the same shelf, and therefore under identical conditions. In the seven experiments (Table IV) a total of 62 precooled animals went into hibernation after an average of 35.5 per cent of their days in the refrigerator while the controls entered hibernation only after 60.5 per cent of their total days in the refrigerator, indicating that precooling caused the animals to hibernate sooner. In the per cent of days in hibernation the effect of precooling was also evident, but not to the same extent. The precooled animals hibernated 41.7 per cent of their total days in the refrigerator, while the controls hibernated only 26.0 per cent.

TABLE IV

Precooling Experiments

Date	Precooled (Experimental) Animals			No. of days	Controls		
	No. of animals	Per cent days before hibernation	Per cent days in hibernation		No. of animals	Per cent days before hibernation	Per cent days in hibernation
1927							
Jan. 1	9	34	43	15	9	43	29
Jan. 12	10	42	44	13	10	75	20
Jan. 27	7	29	40	15	8	72	15
Feb. 15	10	41	30*	14	10	90	8*
1928							
Mar. 6	7	41	24*	8	9	72	9*
1929							
Nov. 7	9	35	43†	11	9	29	51†
Nov. 16	10	20	63	6	9	36	48
Averages‡		35.5	41.7			60.5	26.0

Below are those experiments in which less than three animals in either group (experimental or control) hibernated.

1927							
Mar. 2	9	81	8	14	10	93	3
Apr. 3	9	93	2	18	10	84	13
Apr. 27	6	100	0	7	6	100	0
1930							
Feb. 4	9	98	2	7	9	70	14

* Note low percentage of hibernation in February and March, yet the precooled animals entered hibernation much sooner than the controls.

† Both groups went into hibernation very quickly in this experiment. Controls had been partly precooled, as the room temperature fell to 21° C. or 13° C. each night. Experimentals were in a temperature over night which fell to 10–20° C.

‡ Averages from another table in which each animal is counted equally. Not an average of the group averages given above.

The difference in days *before* hibernation of the two groups was quite significant (D/PE = 6.48) and that for days *in* hibernation was fairly significant (D/PE = 3.88). As the influence of the precooling would wear off in a few days after placing the animals in the refrigerator, more importance should be attached to the data of days *before* hibernation than those of days *in* hibernation.

It should be noted that precooling was conducive to increased hibernation in the months of November to January, with one exception in which the controls had been precooled slightly through inability to control the temperature of the animal house. Precooling was less or not at all effective in the late winter and spring months, the period in which the animals gave indications of increased sexual activity by enlargement of the testes, scrotum and penis in the male and by enlargement of the vulva in the female. While exact comparisons are difficult to make between hibernation at the different seasons, it may be stated that observations over a period of years indicate that spring is not as good a season for hibernation as is the summer.

These experiments would indicate that one cause of more hibernation in the winter than in summer in the laboratory is sometimes a physiological condition produced by external conditions such as intermittent cold which aids in the production of hibernation, providing other conditions are moderately favorable to entrance into hibernation. Intermittent cold may not play a great part in nature, since the daily range is not great between six inches and one foot in the ground (Bouyoucos, 1913, 1916; Sweezy, 1903, McColloch and Hayes, 1923), where most of the hibernating nests occur (Johnson, 1917). However, a daily range of 10° F. or more was frequently noted at the six-inch level by Bouyoucos (1916) in loam and clay in September, but the range was only about half as great in October, when the soil temperature was falling gradually. It seems probable, however, that the gradual cooling of the soil would have a similar effect to that of intermittent precooling in that it would prepare the animal for hibernation. Whether precooling or gradual cooling would eliminate mortality in hibernation in summer cannot now be stated, but general observations point in that direction. It should also be noted that Payne (1927) has found that preliminary cooling as well as dehydration enables insects to withstand more severe and longer periods of cold.

Castration

Since ground squirrels become sexually active very soon after they come out of hibernation and in the animal house begin to show enlargement of the external genitalia in late winter, it was thought that waking from hibernation in the spring was possibly induced to some extent by the increasing activity and internal secretion of the gonads. To test this possibility, a number of male ground squirrels were castrated and after a time were placed in a refrigerator with uncastrated controls. Some animals were used several times. Each experiment usually lasted two weeks. In the first four experiments, consisting of 3 to 5 castrated animals with the same number of controls, from May to July, 1926, there was no hibernation in either castrated or control (uncastrated) animals. From July to December, 1926, five more experiments, and in August and September, 1927, two more experiments were performed in which there was an average of 34.5 per cent of days in hibernation in the 48 castrated males and 35.3 in the 46 uncastrated males, showing no effect of castration on hibernation in the summer, fall, and early winter.

TABLE V

Effect of Castration upon Hibernation in Late Winter and Early Spring

No. of animals	Experimental (castrated) male ground squirrels				Control (uncastrated) male ground squirrels		
	Per Cent days before hibernation	Per Cent days in hibernation	Date begun	Days in refrigerator	No. of animals	Per Cent days before hibernation	Per Cent days in hibernation
			1927				
6		52	Jan. 13	28†	6		43
6		82	Feb. 11	13†	6		37
4		36	Feb. 24	70†	4		13
4	42	43	Mar. 22	50	4	92	6
			1928				
3	0	78	Jan. 30	15	2	25	51
2	32	47	Mar. 8	19	1	28	51
1	100‡	0	Apr. 16	12	1	100‡	0
Av. 2.5	44	42		24	2	61	27
Average of individual records	27.6	55.6				46.2	33.6

† Continuation of a previous experiment.

‡ Animals thin, a condition tending to prevent hibernation.

Experiments performed from January to March, 1927, and January to April, 1928, showed an inhibitory effect of the gonads upon hibernation at this time of year (Table V). The castrated males showed 55.6 per cent days in hibernation, whereas the controls showed only 33.6 per cent days in hibernation in this experiment. The difference is probably significant ($D/PE = 3.52$) because in other experiments normal males have not hibernated much at this time of the year and because a castration experiment with females at this time of year resulted similarly. In five experiments (January to March), with an average of 3 animals in each, the spayed females hibernated 45 per cent and the normal females only 30 per cent of the days in the refrigerator. The spayed animals were in the refrigerator 34 per cent of the total days in the refrigerator before they hibernated whereas the controls were in the refrigerator 45 per cent of the total days before they hibernated. As the number of females is small and the difference not so striking as in the case of the males, these data were not treated

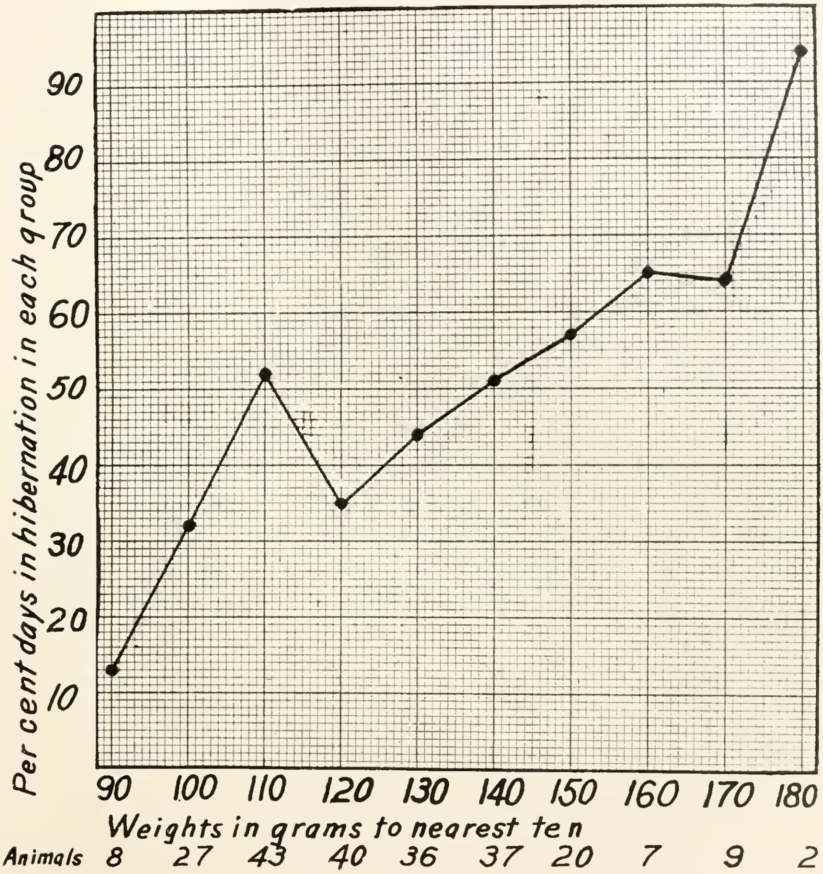

FIG. 1. Graph showing relation of weight to tendency to hibernate in ground squirrels. The bottom row of figures gives the number of animals having the weight given directly above.

statistically. They lend support, however, to the data on the males as the results point in the same direction. It is not improbable that the increased sexual activity which is apparently in part responsible for lack of hibernation in spring is in turn caused by increased activity of the anterior pituitary. Work (unpublished) on the relation of this gland to hibernation has been in progress at this laboratory since 1926.

Weight and Hibernation

As weights were taken before and after each experiment in the refrigerator, it was possible to use existing data for a determination of the relationship between weight and hibernation. From these data the graph in Fig. 1 was made. Thirty-eight heavy animals weighing between 146 and 185 grams hibernated 62 per cent of the total days in the refrigerator, while 118 light animals weighing between 86 and 125 grams hibernated only 39 per cent of the total days in the refrigerator. Treated statistically, this is a significant difference (D/PE = 5.7). The number of days before hibernation took place was only 16 per cent of the total days in the refrigerator in the case of the heavier animals but was 41 per cent in the case of the leaner animals. This difference is also significant (D/PE = 8.1). It is to be seen, however, that there was not a perfect gradation of per cent of days of hibernation (Fig. 1) from the lightest to the heaviest animals; thus 43 animals weighing between 106 and 115 grams (given as 110 grams in the graph) hibernated more than another group of 40 animals weighing 10 grams more, but a predominance of high percentages of days *in* hibernation is found in the heavier groups and there is a predominance of high percentages of days *before* hibernation in the lighter groups.

SUMMARY

Controlled experiments performed on large numbers of ground squirrels showed that individuals starved or given very limited rations went into hibernation sooner and hibernated longer than those receiving an abundance of food. Moderately bright illumination with electric light did not affect the tendency to hibernate in any way. Confined air and precooling the animals nightly both aid in the production of hibernation. Castration does not influence hibernation except during the breeding season in the spring when castrated males hibernate more readily than normal males. The few spayed females used also hibernated more than the normal females in the spring. Obesity is favorable to hibernation.

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TIME OF DEVELOPMENT OF THE DIFFERENT SEXUAL FORMS IN *DROSOPHILA MELANOGASTER*

TH. DOBZHANSKY

California Institute of Technology, Pasadena

In the progeny of a triploid female crossed to a normal diploid male there appear six different sexual types. These are the diploid females and males, triploid females, intersexes, and the two kinds of so-called supersexes, namely superfemales and supermales. As shown by Bridges (1921, 1922) these sexual types are due to variations in the ratio between the number of X-chromosomes and the number of sets of autosomes present in cells of a given individual. According to Bridges the different sexual types have the following ratios:

	Number of X-chromosomes	Number of Sets of Autosomes	Ratio
Superfemale	3	2	1.50
Diploid female	2	2	1.00
Triploid female	3	3	1.00
Intersex	2	3	.67
Male	1	2	.50
Supermale	1	3	.33

Most of the characters of the flies change in this series of sexual types hand in hand with the change of the sex-determining ratio. There are, however, some characters of the flies which seem to be independent of the sex-determining ratio. Among such characters may be listed the size of the cells, which is correlated with the amount of chromosomal material present in their nuclei (Dobzhansky, 1929). Another character of this kind is the length of the development period, to the study of which the present paper is devoted.

The length of the development period has been studied previously in only the diploid males and females (Bonnier, 1926). According to Bonnier, the development of males takes a slightly longer time than that of females. At 25° the development of a female takes 227.98 hours on the average, and the development of a male 232.24 hours. At 30° the corresponding figures are 178.10 and 187.63 hours.

The technique of the present study was as follows. Batches of from twenty to thirty wild-type triploid females were kept together with an approximately equal number of males for two days to ensure fertilization. The flies were then transferred to the ordinary culture-bottles provided with a standard amount of food, but without filter-paper, which is regularly placed in each culture-bottle. Here the flies were allowed to lay eggs during a two-hour period, and after that were again transferred to fresh bottles without paper, and again allowed to lay eggs during another two-hour period. The above procedure was repeated from five to six times with each batch of flies. All the transfers from bottle to bottle were performed without etherization of the flies, because females of *Drosophila* are known to interrupt the egg-laying for several hours after being etherized. The bottles with eggs secured by this method were placed in an incubator and kept at 27°. As soon as the mature flies began to emerge, bottles were examined every two hours. In young flies the determination of the sexual type is sometimes difficult; in cases of doubt, flies were kept alive until the sex could be determined with certainty. Only those bottles which produced more than 20 but less than 60 flies were taken into account (the average number of flies per bottle was 38.5). All other bottles were

TABLE I

Time of Emerging from the Pupæ

Time in Hours	Diploid Females	Triploid Females	Males	Super-females	Inter-sexes	Super-males
183	1	—	—	—	—	—
185	14	2	—	—	—	—
187	19	3	2	—	—	—
189	16	4	2	—	—	—
191	35	6	6	—	—	—
193	25	3	2	—	—	—
195	23	—	5	—	—	—
197	25	2	2	—	—	—
199	26	—	9	—	—	—
201	29	5	8	—	—	—
203	29	2	4	—	—	—
205	29	7	26	—	—	—
207	21	6	10	—	—	—
209	22	11	9	—	—	—
211	7	7	7	—	—	—
213	7	6	5	—	—	—
215	4	1	2	—	—	—
217	1	—	1	—	—	—
219	1	—	2	—	—	—
221	—	—	—	—	—	—
223	—	—	—	—	1	—
225	1	1	—	—	1	—
227	—	2	—	—	—	—

TABLE I (*Continued*)

Time in Hours	Diploid Females	Triploid Females	Males	Super-females	Inter-sexes	Super-males
229	1	—	—	—	2	—
231	—	—	—	—	1	—
233	—	—	1	—	3	—
235	5	1	3	—	5	—
237	—	—	—	—	2	—
239	—	—	—	—	13	—
241	—	—	—	—	12	—
243	—	—	—	—	10	—
245	—	—	—	—	8	—
247	—	—	—	—	7	—
249	—	—	—	1	6	—
251	—	—	1	—	9	1
253	—	—	—	1	12	—
255	—	—	—	—	24	—
257	—	—	—	—	18	2
259	—	—	—	—	22	—
261	—	—	—	—	20	1
263	—	—	—	—	10	1
265	—	—	—	—	11	1
267	—	—	—	—	5	1
269	—	—	—	—	4	—
271	—	—	—	—	3	2
273	—	—	—	—	3	1
275	—	—	—	—	2	1
277	—	—	—	—	2	1
279	—	—	—	—	4	—
281	—	—	—	—	6	—
283	—	—	—	—	4	—
285	—	—	—	—	1	—
287	—	—	—	—	—	3
289	—	—	—	—	2	1
291	—	—	—	—	1	—
293	—	—	—	—	—	—
295	—	—	—	—	—	—
297	—	—	—	—	—	1
Total	341	69	107	2	234	17

discarded, since overcrowding of food with larvæ might slow down the development, especially of the weaker types, and bottles producing too few flies usually have the food in poor condition.

The technique just described gives a reasonable degree of certainty that all the flies developed under similar external conditions and that the length of the development period of each individual fly is determined with an error not exceeding ± 1 hour. The material gathered in this way is presented in Table I. As seen from this table, 770 flies were obtained. Among them there were 44.3 per cent diploid females, 9.0 per cent triploid females, 13.9 per cent males, 30.4 per cent intersexes, 0.3 per cent superfemales and 2.2 per cent supermales. This is about the normal frequency of the different sexual types in the progeny of triploid females. In another experiment, in which offspring were obtained from individual triploid females kept in the bottles until their progeny appeared, there were 50.8 per cent diploid females, 6.4 per cent triploid females, 7.9 per cent males, 32.3 per cent intersexes, 0.2 per cent superfemales and 2.4 per cent supermales. (The total number of flies in this experiment equals 8796 flies; the average number of flies per bottle equals 71.6).

Consideration of the data presented in Table I shows at once that the different sexual types do not develop equally fast. The six known types may be divided roughly into two groups. The development of the diploid and triploid females and males takes from 185 to 219 hours, and only a few individuals require longer than that period. On the other hand, intersexes, superfemales and supermales begin to hatch after 220 hours. That is to say, there exists a short period of time when scarcely any flies emerge in the cultures.

TABLE II

The Length of the Development Period (in hours) of the Different Sexual Types

	Mean Value	σ	C	n
Diploid females	199.24 ± .49	9.12	4.5	341
Triploid females	203.83 ± 1.29	10.72	5.3	69
Males	205.20 ± .98	10.08	4.9	107
Intersexes	255.76 ± .82	12.52	4.9	234
Superfemales	251.00	—	—	2
Supermales	272.72 ± 3.12	12.90	4.7	17

The results of the statistical treatment of the material presented in Table I are shown in Table II. The development of the diploid females takes the shortest time as compared with other sexual types. The development of males takes 5.96 ± 1.05 hours longer than that of the diploid females. This difference is statistically significant and is approximately equal to that obtained by Bonnier in his experiments. The absolute values for the length of the development of diploid females and males are also in good agreement with Bonnier's data (one must, of course, take into account that the temperature used in the present experiments is intermediate between the two temperatures used by Bonnier).

The figure obtained for the length of the development of triploid females is intermediate between the figures obtained for diploid females and males, but the differences are not statistically significant in either case. It may be concluded that the development of the triploid females takes approximately as much time as the development of the diploid females and males, and far less than the development of the intersexes and the supersexes.

The development of the intersexes takes 255.76 ± .82 hours on the average. This figure is unquestionably different from those obtained for females and males. Although the figure for the superfemales (251 hours) is based only on two individuals, it may be taken for certain that the length of the development of the superfemales is of the same order of magnitude as that of the intersexes and the supermales. This has been proved in a different experiment in which superfemales were obtained in greater number together with diploid females and males (this experiment deals with the progeny of attached-X females crossed to normal males). In this experiment superfemales appeared in the bottles only in the later counts, when the number of diploid females and males hatching in a given interval of time was already declining. The length of the development of the supermales (Table II) is significantly greater than that of the intersexes. That is to say, the supermales develop more slowly than any of the other sexual types known.

It may be concluded that the length of time of development does not show a correlation with the ratio between the number of X-chromosomes and the number of sets of autosomes, the ratio which is known to determine the sexual type. The types studied may be divided into two groups: the balanced and the unbalanced types. The balanced types are those which have the sex-determining ratio equal to that observed in diploid females or males. Beside the diploid females and males only the triploid females belong to this group. Their development takes a relatively short time. The unbalanced types are the intersexes and the supersexes; their development takes a longer time.

SUMMARY

1. The different sexual types known in *Drosophila* are characterized by different lengths of their development periods. Diploid females develop more quickly and supermales develop more slowly than the other sexual types, which are intermediate between these two extremes (see Table II).

2. There is observed no correlation between the length of development and the sex-determining ratio (*i.e.*, the ratio between the number of X-chromosomes and the number of sets of autosomes present in the cells of a given individual).

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THE GLOMERULAR DEVELOPMENT OF THE VERTEBRATE KIDNEY IN RELATION TO HABITAT

E. K. MARSHALL, JR. AND HOMER W. SMITH

(From the Departments of Physiology, The Johns Hopkins School of Medicine, Baltimore, and Bellevue Medical School, New York University, New York)

Some twenty-five species of fishes, belonging to twelve families, are known to possess nearly or completely aglomerular kidneys (Marshall, 1929, and this paper). It has been suggested that this aglomerular condition is related to the peculiar water cycle associated with a marine habitat (Smith, 1930). In fresh water a large quantity of pure water is absorbed probably by way of the oral membranes, and excreted in large part, if not entirely, by the kidneys; whereas, in sea water a relatively small quantity of sea water with its contained salts is absorbed from the gastro-intestinal tract, and a large fraction of the absorbed water is excreted extra-renally. Thus a persistent oliguria relative to fresh-water forms occurs in marine fish, and this oliguria is held to be the cause of the glomerular degeneration.

In this view glomerular development should be related to water excretion in the vertebrates generally, and we wish to present evidence in this paper that such is the case. This evidence takes the form of three arguments: (1), that the protovertebrate kidney was aglomerular and that the glomerulus was evolved as an adaptation to a fresh-water habitat, (2) that in the lower vertebrates the extent of glomerular development is related to the quantity of water normally excreted by the organism and (3) that in the mammals (and possibly to some extent in lower vertebrates) the primitive water-excreting function of the glomerulus has been secondarily diverted to a filtration-reabsorption system designed to excrete waste products without the loss from the body of excessive quantities of water.

The Protovertebrate Kidney

The only information available on the nature of the protovertebrate kidney is that which may be obtained from the embryonic development of the lower vertebrates. There are no essential differences in the development of the pronephros of the cyclostomes, fishes and Amphibia, but we may suppose that the cyclostomes approach most closely to the primitive condition. Of these the developmental history is best known in the case of *Petromyzon* which has been studied by Wheeler (1899), Hatta (1900–01) and Inukai (1929). Additional information on the vertebrate pronephros is given by Felix in Hertwig's Handbuch (1906).

The pronephros of the lamprey at an early stage consists of six tubules on either side, formed from mesoderm and opening by means of nephrostomes into the unsegmented body cavity just behind the branchial region. In a later stage some of the tubules at the cranial and caudal end degenerate while the remainder (according to Inukai from 3 to 6 pairs) develop and function for a considerable time during the larval period. These pronephric tubules communicate with the pericardial cavity, which at about this time has been cut off from the peritoneal cœlom. Hatta regards the segmental duct as being formed by a series of abortive pronephric tubules in about twelve somites lying posterior to the eighth somite. After the formation of paired tubules, further development of the pronephros consists in the lengthening and convolution of the tubules, and the formation of a blood supply and glomerulus.

The blood vessels which supply the pronephros acquire definite form in a comparatively late stage of development. The glomerulus or glomus is lobulated, supplied by at least three branches of the aorta and hangs free in the pericardial cavity.

Hatta comes to the conclusion that in the ancestors of *Petromyzon* the pronephros once extended over all the body segments from the branchial to the cloacal region, the tubules opening to the exterior in each segment. The tubules of the posterior region were later converted into the segmental duct which first opened to the exterior and then into the cloaca.

In teleosts, where several originally distinct pronephric tubules have fused to form the pronephric chamber and a single tubule, the glomerulus is evolved after the separation of the pronephric chamber from the cœlom has taken place. Therefore, the pronephric tubule is aglomerular and unconnected with the cœlom for a considerable time during development (Emery, 1882; Felix, 1906). The glomerulus is here an inner glomerulus and consists of an invagination of a capillary tuft into the dilated end of the tubule which forms the pronephric chamber. Besides this condition and that observed in *Petromyzon,* there is a third and more usual type of pronephric development where the glomerulus is formed by a more or less complete abstraction of the true body cavity with an outer glomerulus or glomus in a pronephric chamber. Of these three types it would appear that that of the lamprey is the most primitive. Here, the glomerulus is quite distinct, separate from, and formed much later than the pronephric tubules.

That the glomerulus was developed after and secondarily to the pronephric tubules in the early vertebrates is certainly suggested by the above discussion. It is borne out by many other considerations. In invertebrates, nothing resembling a glomerulus occurs, but we find as excretory organs various types of tubules with glandular epithelium. The mesonephros of teleosts is stated to be aglomerular in young embryos (Audigé, 1910). In certain mammals (*e.g.,* rat and mouse) the mesonephros never develops glomeruli (Bremer, 1916). In most vertebrates the mesonephros is usually first formed of segmental tubules connected to the cœlom by nephrostomes. Later the glomerulus develops and we have a tubule containing a typical Malpighian body with its glomerulus, but still connected to the body cavity by a nephrostome, as in the adult Urodeles. However, the tubule in these forms usually loses its connection with the cœlom (Widersheim, 1906). Borcea (1906) states that in the development of the mesonephros of certain elasmobranchs (*e.g., Raia*) the segmental tubules lose their connection with the body cavity before the formation of glomeruli, thus paralleling the development of the pronephros of teleosts.

We may infer, then, that at some period the protovertebrate kidney was aglomerular and that it probably consisted of a series of tubules communicating with the cœlom by means of nephrostomes, the tubules either opening separately to the exterior or opening into a common duct. The tubules extended over a greater portion of the body than is the case with the pronephros at present. The tubules were not simple conduits, but their epithelium was glandular in structure and they may have both reabsorbed substances from the cœlomic fluid passing through them and added to this fluid by secretion. That secretion was a function of the primitive tubules is suggested by the fact that the tubular epithelium is derived from cœlomic epithelium and by the fact that crystals have been found in the lumen of the blind pronephric tubule of the embryo trout before the glomerulus has developed (Nussbaum, 1886.)

The cœlom seems to have been the original excretory organ, being connected with the exterior by means of pores, (*c.f.,* abdominal pores of cyclostomes and elasmobranchs) or by tubules opening into the cœlom by means of nephrostomes. Felix (1906) says, " als primitivestes Harnorgan haben wir fraglos die Leibeshohle selbst anzusprechen." The excretory function of the primitive cœlomic epithelium is indicated by the connection of both pronephros and mesonephros with its cavity, by the existence of abdominal pores in cyclostomes and elasmobranchs, and by the composition of the cœlomic and pericardial fluids of the latter (Smith, 1929b).

With the development of glomeruli, the nephrostomal connection of the tubule with the cœlom is usually abolished. The nephrostomes draining the cœlom may be secondarily diverted to empty into the venous system (Amphibia), into lymphoid spaces (elasmobranchs) or they may disappear completely.

Ever since Bowman published his original theory of urinary secretion in 1842 the glomerulus has been assigned the rôle of eliminating most of the water of the urine. All subsequent theories have taken this premise as their starting point. When this developmental and functional evidence is coupled with the evolutionary history of the lower vertebrates it appears probable that the glomerulus was evolved in response to the need for an easy means to excrete large quantities of water.

There is much evidence in the geological nature of the strata in which the early fossil vertebrates are found to indicate that these animals were inhabitants of fresh or slightly brackish water (Chamberlain, 1900; Barrell, 1916; O'Connell, 1916; Kiâer, 1924; Geikie, 1903; Woodward, 1900; Grabau, 1921; Hussakof and Bryant, 1918; Patten, 1912; Stromer, 1920). The subject presents many palæontological and geological difficulties and must still be considered a controversial one. According to Barrell (1916) with slight extensions based on the observations of other palæontologists and geologists, it would appear that the Silurian and Devonian ostracoderms and fishes were inhabitants of the continental rivers and fresh-water lakes. From some unknown relatives of these fresh-water forms there were evolved the Devonian elasmobranchs, dipnoans and ganoids and later the Carboniferous Amphibia. The elasmobranch fishes migrated to the sea toward the middle or late Devonian and, though frequently invading fresh water in subsequent times, this sub-class is predominantly marine today. The ganoid fishes may have invaded the sea to some extent in the Devonian, but so far as those forms which were ancestral to the recent teleosts are concerned, the permanent assumption of a marine habitat appears to date more properly from Carboniferous or even Mesozoic times. Judging in part from the historical record and in part from the life habits of the recent fishes, it would appear that the recent dipnoans, ganoids and many primitive teleosts have had a more or less continuous fresh-water history since the early Palæozoic period, while the recent marine teleosts may be assumed to have lived in pelagic or deep ocean waters only through Mesozoic and Tertiary time.

The composition of the sea in past ages is unknown, but it may be inferred that the salinity in the Devonian period was at least half, if not three-quarters, of what it is at present. It is certain that the salinity of the ocean waters throughout the Tertiary has been great enough to impose upon the marine teleosts the same osmotic restraints in regard to the absorption and excretion of water and salts that characterize this habitat today.

When these separate lines of evidence are brought side by side it is a logical deduction that the glomerulus was evolved in some early Palæozoic chordate to enable the organism to excrete readily the large quantity of water which was absorbed along the osmotic gradient existing between its blood and its fresh-water environment. This glomerulus represented simply an advantageous juxtaposition of the blood-vascular system to the already existing tubular system draining the excretory cœlom.

So long as the organism remained in fresh water (dipnoans, ganoids and teleosts) or in intimate dependence upon it (Amphibia), this excretory arrangement persisted; but with the secondary assumption of a marine habitat (teleosts) where the osmotic gradient was reversed and the water excretion reduced, or with the assumption of terrestrial life in which water conservation became a necessity (arid-living reptiles and birds), the organism no longer needed and could no longer economically use this primitive water-excreting mechanism. There was thus a need to either (*a*) discard or reduce the glomeruli or (*b*) amend their primitive function by adding distally a more efficient mechanism for the reabsorption of water. The first process appears to be occurring in the marine teleosts and in the reptiles. In the mammals and possibly to some extent in the birds, on the other hand, the addition of the loop of Henle has permitted the reabsorption of water against the osmotic pressure of the metabolites in the urine; consequently in these the glomeruli, although still very active as filters, have become secondarily incorporated into a filtration-reabsorption system which permits the excretion of waste products without the excretion of excessive quantities of water.

In substantiation of this thesis we wish to present here a description of the glomerular development in some fresh-water and marine fishes and in a few other vertebrates. No attempt has been made to interpret the structure of the kidney in larval forms because nothing is known at present about their water excretion or osmotic relationships. The marine cyclostomes and elasmobranchs possess blood which has about the same osmotic pressure as sea water, and such limited knowledge as we have of these animals indicates that the water cycle in them is quite different from what it is in the marine teleosts. Until more information is available on these points we cannot expect to fit these sub-classes into the present hypothesis.

The Glomerular Development in Adult Vertebrates

In examining different animals we have used the following criteria in judging glomerular development:

1. Reduction in the number of glomeruli and presence of aglomerular tubules (here the extreme stage is an aglomerular kidney).
2. Very small size of the glomeruli.
3. Lack of good vascularization in the glomerular tuft, whether due to the presence of excessive amounts of connective tissue or to lack of capillary branching.

When only one of these conditions occurs one cannot draw definite conclusions, but if two or all conditions are present, it seems safe to conclude that glomerular development is poor.

The Higher Fishes.—Our observations have been most extensive in the heterogeneous group of teleostean fishes. We have collected a large series of data here in order to test our hypothesis.

The kidney of teleosts has not been extensively or intensively studied. It appears that wide variations in its structure can occur. What is known at present of the structure of its renal tubule has been summarized in a recent paper (Marshall, 1930). The long paper by Audigé (1910) is the most complete on teleost kidneys. Following Hyrtl (1851), Audigé divides the kidney into an anterior, middle, and posterior kidney. He further states that the posterior kidney is a metanephros, has well developed large glomeruli with branching capillary tufts, and that no aglomerular tubules occur; that the middle kidney has none, few, or many glomeruli, which are small and consist of a single coiled capillary; and that the anterior kidney in most adult forms consists entirely of lymphoid tissue. On this basis one can divide the teleosts into two groups, those with and those without a posterior kidney. We made such a division from all the data we could collect from the literature. Although in general the marine teleosts fell into the group without posterior kidney (poor glomerular development);

and the fresh-water fish into that with posterior kidney (good glomerular development), there were many exceptions. Our subsequent histological study of sections from the kidneys of many teleosts convinced us that a classification on the basis of the examination of sections was much more accurate than the above, and hence we omit this preliminary classification.

We have divided the higher fishes into four groups on the basis of glomerular development as shown from a study of sections of the kidney. These are as follows:

Group I. Kidneys having frequent glomeruli which are medium or large and invariably well vascularized. This group presents extremely good glomerular surface.

Group II. An intermediate group. Kidneys may have frequent glomeruli which are very small, or may have few glomeruli which are small or fair sized. Kidneys never have both frequent and medium sized glomeruli.

Group III. Kidneys having infrequent glomeruli which are small, poorly vascularized and which may show signs of degeneration. This group presents extremely poor glomerular surface.

Group IV. Aglomerular kidneys. In this group are included *Lophius,* where the very few glomeruli present are non-functional in the adult (Grafflin, 1929), and several species described by Guitel (1906) in which a single large glomerulus occurs in the persistent pronephros, but in which the remainder of the kidney (mesonephros) is aglomerular.

There would appear to be little error involved in placing a species into groups I and IV, but some selection is necessary to determine whether a species should go into group II or III and to a less extent as to whether it should go into group I or II. Any questionable case has been put in the intermediate group II, so that it is possible that some species in group II should have been in group I or III.

The following table gives the groups as defined above and the average size of the renal corpuscles in sections of kidney fixed and treated in the same way.[1] The habitat of the species studied is given in

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[1] Measurements of the size of the renal corpuscle in fixed sections are undoubtedly too small due to shrinkage. Another objection to measuring glomeruli in sections is the difficulty of always measuring a section cut through the middle. This error will affect the larger glomeruli more than the smaller. Distortion of the shape of a Malpighian body also occurs in sections. This has led us to average the two diameters measured in ten corpuscles and give a single figure as representing the size. Using our measurements only to indicate whether the glomeruli are small, medium or large would seem to be justified in spite of the many unknown factors which must be considered in comparing different animals. We have worked only with adult animals.

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Table I

Glomerular Development and Habitat of Higher Fishes

Species	Family	Common Name	Habitat	Size of Renal Corpuscle in Micra
Group I				
Protopterus æthiopicus Heckel	Lepidosirenidæ	Lung-fish	F	153
Polypterus senegalus Cuvier	Polypteridæ		F	89
Amia calva Linnaeus	Amiidæ	Bowfin	F	82
Plecostomus plecostomus (Linnaeus)	Loricaridæ		F	73
Cyprinus carpio Linnaeus	Cyprinidæ	Carp	F	82
Carrassius auratus (Linnaeus)	Cyprinidæ	Goldfish	F	62
Catostomus commersonii (Lacépède)	Cyprinidæ	Sucker	F	60
Ameriurus nebulosus (LaSueur)	Siluridæ	Catfish	F	99
Morone americana (Gmelin)	Serranidæ	Silver perch	E	63
Ambloplites rupestris (Rafinesque)	Centrarchidæ	Bass	F	86
Enneacanthus gloriosus (Holbrook)	Centrarchidæ	Sunfish	F	76
Perca flavescens (Mitchill)	Percidæ	Perch	F	102
Salmo gairdneri Richardson	Salmonidæ	Trout	E	60
Salvelinus fontinalis (Mitchill)	Salmonidæ	Trout	E	75
Umbra limi (Kirtland)	Esocidæ	Mud minnow	F	106
Esox lucius Linnaeus	Esocidæ	Pickerel	F	81
Anguilla rostrata LaSueur	Anguillidæ	Eel	E	104
Gymnothorax funebris Ranzani	Murænidæ	Moray	M	112
Myoxocephalus octodecim (Mitchill)	Cottidæ	Sculpin	M	81

TABLE I (*Continued*)

Species	Family	Common Name	Habitat	Size of Renal Corpuscle in Micra
Group II				
Copeina guttata (Steindachner)	Characidæ		F	48
Astanyx sp.	Characidæ		F	50
Centropristis striatus (Linnaeus)	Serranidæ	Sea bass	M	52
Hæmulon album Cuv. and Val.	Hæmulidæ	Grunt	M	34
Hæmulon album Cuv. and Val.	Hæmulidæ	Grunt	M	64
Holocentrus ascensionis (Osbeck)		Squirrel fish	M	39
Gadus callarias Linnaeus	Gadidæ	Cod	M	37
Melanogrammus æglefinus (Linnaeus)	Gadidæ	Haddock	M	38
Hemitripterus americanus Gmelin	Cottidæ	Sea Raven	M	65
Chilomycterus schœpfi (Walbaum)	Diodontidæ	Spiny box fish	M	59
Caranx ruber (Bloch)	Carangidæ	Jack	M	33
Sphyræna barracuda (Shaw)	Sphyrænidæ	Barracuda	M	35
Strongylura raphidoma Ranzani	Sphyrænidæ	Hound fish	M	46
Tautoga onitis (Linnaeus)	Labridæ	Tautog	M	56
Scomber colias Gmelin	Scombridæ	Tinker mackerel	M	48
Cryptacanthodes maculatus Storer	Cryptacanthodidæ	Wrymouth	M	94
Pseudopleuronectes americanus (Wal.)	Pleuronectidæ	Flounder	M	50
Poronotus triacanthus (Peck)	Triglidæ	Butter fish	M	51
Stenotomus chrysops (Linnaeus)	Sparidæ	Porgy	M	51
Chætodipterus faber (Broussonet)	Chætodipteridæ	Spade fish	M	40
Balistes vetula Linnaeus	Balistidæ	Trigger fish	M	41
Sebastes marinus (Linnaeus)	Scorpænidæ	Rose fish	M	44

TABLE I (*Continued*)

Species	Family	Common Name	Habitat	Size of Renal Corpuscle in Micra
Group III				
Argyropelecus hemigymnus Cocco	Stomiatidæ		M	
Chauliodus sloanei B. and S.	Stomiatidæ		M	
Gonostoma bathyphilum (Vaillant)	Stomiatidæ		M	
Gonostoma grande (d?)	Stomiatidæ		M	
Strongylura notatus (Poey)	Belonidæ	Needle fish	M	39
Strongylura sp.	Belonidæ	Bill fish	M	37
Cypselurus heterurus (Rafinesque)	Exocœtidæ	Flying fish	M	30
Teuthis hepatus Linnaeus	Teuthidæ	Brown tang	M	27
Sphæroides maculatus (B. and S.)	Tetradondidæ	Puffer	M	38
Lactophrys bicaudalis (Linnaeus)	Ostrociidæ	Shell fish	M	26
Group IV				
Syngnathus dumerilii (Kaup)	Syngnathidæ	Pipe fish	M	None
Syngnathus acus Linnaeus	Syngnathidæ	Pipe fish	M	“
Siphonostoma sp.	Syngnathidæ	Trumpet fish	M	“
Nerophis lumbriciformis (Willoughby)	Syngnathidæ		M	“
Entelurus anguineus (Kaup)	Syngnathidæ		M	“
Hippocampus brevirostris Storer	Syngnathidæ	Sea horse	M	“
Hippocampus guttulatus Cuvier	Syngnathidæ	Sea horse	M	“
Lepadogaster gouanii Bris. de Ban.	Gobiesocidæ		M	“
Lepadogaster bimaculatus (Pennant)	Gobiesocidæ		M	“
Lepadogaster candolli Risso	Gobiesocidæ		M	“
Lepadogaster macrocephalus (d.?)	Gobiesocidæ		M	“
Chorisochismus dentex (Pallas)	Gobiesocidæ		M	“
Gastrostomus bairdi Gill and Ryder	Saccopharyngidæ		M	“
Pterophryne histrio (Linnaeus)	Antennariidæ	Mouse fish	M	“
Opsanus tau (Linnaeus)	Batrachidæ	Toad fish	M	“
Lophius piscatorius Linnaeus	Lophiidæ	Goose fish	M	“

the third column, *F* indicating fresh water, *M* marine and *E* euryhaline (where the fish can live in fresh or salt water). The evidence for the aglomerular nature of the kidneys of group IV is given by Marshall (1929) and that for placing the first four fish in group III by Nusbaum-Hilarowicz (1923). The kidneys of all the remaining fish have been examined in the course of this study.[2] The microphotographs of typical fields in cross sections of the kidney given at the end of this paper illustrate fairly well the differences between these groups (Plates I, II and III).

The relationship of habitat to glomerular development is brought out forcibly by the extremes represented in groups I and IV.

The fishes of group IV are aglomerular and thus represent the extreme condition which we imagine to result from a very low water excretion, namely, the complete absence of all filtering surface as typified by the glomerulus, and the presence only of tubular tissue of a high, cuboidal and secretory nature. The fish included in this group are all marine, and even related species do not migrate into fresh water except in rare instances which we may believe represent secondary invasions of fresh water. (The Syngnathidæ have representatives in Panama which may occasionally be found in brackish to fresh-water streams). They are all end-products or terminal members in the evolution of specialized Teleostei. The Saccopharyngidæ are abyssal or deep water forms, though occasionally the latter may invade brackish water. The remaining families are littoral or pelagic forms, and though some of them (*Opsanus*) may normally invade brackish waters, none is known to migrate into strictly fresh water.

The fishes of group I represent the primitive condition which we imagine existed in the Palæozoic ganoids which were ancestral to all the Teleostei, marine and otherwise. In these the glomeruli are numerous, large, with very flat capsular epithelium and the capillary tuft is finely divided by branching. Here the filtering surface is obviously extensive. These species are with two exceptions (*Myoxecephalus* and *Gymnothorax*) either exclusively fresh-water or euryhaline forms. *Ameriurus, Myoxocephalus, Gymnothorax* and *Plecostomus* are relatively highly specialized forms; the others are primitive types. The Loricaridæ, Siluridæ, Cyprinidæ and Characidæ belong to the Ostariophysi, which is typically a continental and fresh-water order. *Amia* is a primitive ganoid and *Protopterus* and *Polypterus* represent the two most ancient types of surviving fishes.

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[2] The specimens of *Protopterus* and *Polypterus* were collected by H. W. S. in Africa through the favor of the John Simon Guggenheim Memorial Foundation.

In contrasting these groups we note that the members of groups III and IV consist of aglomerular or nearly aglomerular fish, of a generally specialized type, which are exclusively marine; group I consists of well glomerularized fish, of a generally primitive type, which are with few exceptions exclusively fresh-water or which occasionally enter fresh water. It is perhaps significant that all the abyssal marine forms which have been studied fall into groups III or IV while the continental and pelagic forms fall into groups I and II. An abyssal habitat may be taken as evidence of a long marine history. It is to be expected that aglomerular kidneys would more probably occur in fishes which had been marine for a long period of time. The fact that the aglomerular forms are in other respects specialized rather than primitive is in accord with general principles of evolution.

Between the extremes of fresh-water or primitive marine fish, on the one hand, and highly specialized marine fish on the other, it is to be expected that there would occur every degree of glomerular development. That such is the case is shown by the character of the fish which we have placed in the intermediate groups II and III. Group III consists of fish with greatly reduced glomerular development; the glomeruli are small, poorly vascularized and very infrequent. These fish are all marine and specialized forms. They approach quite closely to the members of group IV in general character and in glomerular development. It is possible that among these fish some of the few remaining glomeruli are non-functional.

The members of group II are intermediate in glomerular development and heterogeneous in character. They are mostly marine, and include specialized as well as more generalized forms. It is, of course, in this group that the need of a more quantitative expression of glomerular development is most pressingly felt, but this gap in our knowledge is not the only deficiency; we have no information as to how long these fish have been marine or to what extent they may have migrated from salt to fresh water, or *vice versa,* in the course of teleostean evolution. Without such information a quantitative interpretation of this group is practically impossible.

It may be noted that some instances of secondary invasion of fresh water are clearly evident. Thus in the sub-order Apodes the Saccopharyngidæ, Murænidæ and most of the Anguillidæ are marine; but the common eel, *Anguilla rostrata,* lives in fresh water and only returns to the sea to spawn. It may be inferred, then, that a fresh-water habitat is relatively recent for this species. The deep-sea *Saccopharyngida, Gastrostomus bairdi,* is aglomerular; while *Anguilla* shows the typical fresh-water development of the glomeruli. It is probable that the fresh-water Percidæ and Centrarchidæ and the brackish-water Pleuronectidæ, Tetradondidæ and Cottidæ represent forms derived from marine stocks and secondarily entering fresh-water.

Until more is learned, both about the physiology of these forms and their evolutionary history, a closer interpretation than that attempted above is clearly impossible.

Amphibia.—A large amount of work has been done on the kidneys of the Amphibia, yet there is no evidence that aglomerular tubules ever occur in this class. The glomeruli appear to be numerous, well vascularized and the capsule has a low epithelium. This is in line with the close ecological dependence of the Amphibia on fresh water.

The largest glomeruli of all animals occur in the Urodeles (Hyrtl, 1863). Steinbach (1927) gives measurements of the size of glomeruli from sections of several species of Amphibia. These vary from 86 to 217 micra. Using our technique, we find for the average size of the glomerulus in *Rana catesbeiana,* 115 micra; *Bufo americanus,* 92 micra; *Plethodon cinereus,* 145 micra; *Siren lacertina,* 211 micra; and *Necturus maculosus,* 308 micra.

There are some Amphibia which burrow and remain underground during the drought season (North Central Australia). Sweet (1907) states that these forms take in large quantities of water by the mouth and skin before estivating and excrete and store it in the urinary bladder. The water is reabsorbed from the bladder into the abdominal cavity during estivation and passed by the nephrostomes (of which these species have an exceptionally large number) into the renal veins; in this way the water is used over and over again. This absorption of water from the urinary bladder is in line with the recent observation of Steen (1929) that, when exposed to dry air, frogs may reabsorb all the water from the urinary bladder. Sweet states that in one of these burrowing species (*Chiroleptes alboguttatus*) the glomeruli are very few in number and remarkably small.

But as a class the amphibians are provided with good glomerular surface. It is difficult to compare the separate species because of the wide variation in the size of the animal and of the size of the glomerulus. We have not attempted any quantitative analysis within this class.

Reptiles.—The glomeruli of reptiles are peculiar in that the center of the glomerular tuft consists of connective tissue with capillaries only on the outside (Regaud and Policard, 1903–04; Cordier, 1928). In addition to this peculiarity, the Malpighian corpuscle is rather small. Bowman (1842) gives the diameter of the glomerulus of the tortoise as 106 micra, and of the boa constrictor as 63 micra; Hüfner (1866) gives for *Testudo græca* and *Emys europoca,* 110 micra; Regaud and Policard (1903–04) give for the Ophidia, 110 x 90 and 80 x 60 micra; and Hoffman (1890) gives for the Saurians 45 to 52 micra. Zarnik (1910) reports measurement of the glomeruli of the longest tubules of several species of reptiles from macerated preparations, but since they cannot be taken as average values, they are of no assistance to us here.[3] Cordier (1928) reports the average size of the glomerulus in the Chelonians as 50 micra, in the Ophidians as 80 micra and in the Lacertilians as 50 micra. He made careful reconstructions of the glomeruli of these forms and came to the conclusion that the capillary is never an unbranched vessel as stated by Regaud and Policard (1903–04); but the filtering surface of the glomerulus in Chelonians is fairly good and in Ophidians and Lacertilians extremely poor. Cordier clearly recognizes that this difference is related to the elimination of water. He finds from sections that the decrease in filtering surface of the glomerulus of snakes and lizards is not compensated for by increased number, and relates the poor glomerular development of the former to their solid or semi-solid excrement; and the much better development in Chelonians, to their fluid urine.

Table II summarizes our observations on a few species.

TABLE II

Glomeruli of Reptiles

Scientific Name	Common Name	No. Renal Corpuscles	Size in Micra	Vascularity of Tuft
Pseudemys sp.	Slider Terrapin	Frequent	63	Fair
Caretta caretta (Linnaeus)	Sea Turtle	"	91	"
Alligator mississippiensis (Daudin)	Alligator	"	—	"
Boa imperator Daudin	Boa Constrictor	Infrequent	71	Poor
Liopeltis vernalis (Harlan)	Grass Snake	"	59	"
Phrynosoma cornutum (Harlan)	Horned Toad	"	51	"

Two distinct groups of reptiles can be established: one with fair glomerular surface and fluid urine (Chelonia and Crocodilia); the other with very poor glomerular surface and solid or semi-solid urine (Lacertilia and Ophidia). It is in this last group that Regaud and Policard (1903–04) and Zarnik (1910) have found blind diverticula or aglomerular tubules. Microphotographs of sections from kidneys of the terrapin, horned toad, and boa constrictor are given at the end of this paper (Plates III and IV).

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[3] As a rule no statement is given as to how the measurements were made (sections, macerated tissue, or fresh material). This probably accounts for many of the wide discrepancies in the figures. The size of the animal from which the kidneys were removed would also be an important factor.

Birds.—In the bird's kidney we find the beginning of the loop of Henle. Many tubules occur without the loop, however, and these have a short intermediate segment similar to that of reptiles. Many other tubules show transitional stages (Huber, 1917; Feldotto, 1929). The glomeruli of birds' kidneys are stated to be the smallest known; they are, however, no smaller than some occurring in marine teleosts. The capillary loop of the glomerulus is described by some observers as unbranched and by others as only slightly divided. Bowman (1842) states that the " Malpighian vessel is a coiled ampulla "; Hyrtl (1863) says that in spite of the extremely small size of the glomerulus, the vessel is not simple but divides in the smallest glomeruli into 2, and in the largest into 7 or 8 branches. Standfuss (1908) also remarks on the small number of capillary loops in the glomeruli of birds.

Bowman gives the diameter of the glomerulus of the parrot as 59 micra and Hüfner (1866) gives for the dove 44 x 35 micra. Von Mollendorff (1922) has measured the glomeruli of several birds in macerated preparations. He finds average diameters of 48 micra for the pigeon, 38 micra for the ring-sparrow, 28 micra for the house-sparrow, and 24 micra for the finch. We have examined sections of the kidneys of the chicken and pigeon, and find the average diameter of the Malpighian bodies to be 70 micra in the former and 50 micra in the latter. A curious fact about the glomeruli of these birds, which so far as we know has not been noted before, is that the central part of the glomerular tuft resembles the reptilian glomerulus in its lack of capillaries. There is, however, instead of connective tissue a central core of dense syncytial-like tissue. A microphotograph of a section from a chicken kidney is given (Plate IV).

It is obvious that the bird's kidney shows glomerular degeneration, as indicated by the very small size and poor vascularization of the glomeruli, and by the replacement of the central portion of the tuft by syncytial tissue. It is improbable that increased number of glomeruli can offset this reduction in filtering surface.

Mammals.—So far as is known, the tubules of mammalian kidneys are supplied with large, well vascularized glomeruli. Bowman (1842) gives the average size of the glomerulus of a number of mammals ranging from 100 micra in the mouse to 362 micra in the horse. Many subsequent observers give measurements for different species (See Vimtrup, 1928, and v. Mollendorff, 1929). Thus the smallest mammalian glomerulus can be considered large in comparison with those occurring in many marine teleosts, reptiles and birds.

It is in the mammalian kidney that the loop of Henle attains for the first time its full development. This fact is significantly associated with the marked hypertonicity which mammalian urine can exhibit in comparison with the blood plasma (Crane, 1927) and suggests that the glomerular function in these animals has been almost completely modified from its primitive water-excreting function. It is interesting to note that in the primitive *Echidna,* the kidney resembles that of the reptile as much as that of the higher mammals, and that here the glomerular development is stated to be less than in other mammals of the same size (Zarnik, 1910). Much more work, however, will have to be done before mammals can be compared in a quantitative way with other animals or among themselves.

Discussion

The idea that the development of the glomerulus is an adaptation to water excretion by the kidney agrees well with the facts which we have presented.

In the diversified group of higher fishes, we find convincing evidence of the influence of a marine habitat on the development of the glomerulus. In the snakes and lizards as well as the birds, where water conservation is so important that a solid or semi-solid urine is eliminated, the unquestionable reduction in glomerular surface is entirely in accord with what might be predicted.[4] The good glomerular development of the fresh-water fishes and the Amphibia is quite in line with the general thesis. Difficulties arise in attempting to compare different animals with one another in a quantitative way. It is essential to know the number of glomeruli in the kidneys, and also the surface area presented by the capillaries of an average glomerulus. One can obtain the number by accurate counts, but there is no accurate method for obtaining the surface area of a glomerulus and more particularly of the variable capillary tuft. It appears to be generally true that the smaller the glomerulus, the less the capillary tuft is broken up by branching, but the amount of space in the glomerular tuft not occupied by capillaries also seems quite variable in different animals. The recent careful investigation of the surface of a human glomerulus by Vimtrup shows how erroneous are the estimates of glomerular surface used by Putter (1926) and von Mollendorff (1922).

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[4] In the reptiles and birds the urine coming from the ureters is fluid, and the final reabsorption of water takes place in the cloaca. Quite different figures have been given for the ureteral urine flow of the bird, none of which has been determined under strictly normal conditions. Gibbs (1928, 1929*b*) finds that extremely concentrated urine may come from the fowl's ureters. The fact that the ureteral urine is always extremely concentrated as regards waste products when compared to the urine of fresh-water fish and Amphibia, may be taken as supporting our hypothesis.

Even with the number of glomeruli in a kidney determined, there is no exact method of correlating the number in different animals of widely varying size. No data exist to show on what basis such a comparison should be made. All one can do at present is to compare the number of glomeruli in the kidneys of animals of approximately the same size on the basis of body weight and then judge the relative glomerular surface by the average diameter of a glomerulus. An attempt at a more quantitative comparison of glomerular surface is in progress for fish, reptiles and birds of various sizes, and a further quantitative interpretation will be deferred until this work has been completed. For mammals (von Mollendorff, 1929) and Amphibia (Steinbach, 1927) the number and size of the glomeruli have already been determined for many species.

In conclusion, it may be remarked that the relative amounts of a substance eliminated by the glomerulus and tubule will depend on the glomerular development of the animal. If we accept the filtration theory of glomerular function, it is obvious that in eliminating water through the glomerulus, diffusible plasma constituents must also be eliminated and excreted unless reabsorbed by the tubule.[5] Looking at this problem from the standpoint of secretion by the tubule, it is clear that where a large amount of fluid is eliminated by the glomerulus, the secretory function of the tubule will be minimal, but where small amounts are eliminated in the case of poor glomerular development, secretion by the tubule will be maximum. This agrees fairly well with what we know about secretion by the tubule in different classes of vertebrates. Thus, in mammals, it has been supposed that filtration by the glomerulus and reabsorption by the tubule play a major rôle in the production of the urine, and that secretion exists only as a relic of a primitive process (Mayrs, 1924; Marshall, 1926). On the other hand, in birds there is now rather conclusive evidence (Mayrs, 1924; Gibbs, 1929*a*) that secretion plays a major rôle in the elimination of uric acid by the kidney. That secretion by the tubule also will play an important rôle in excretion by the reptilian kidney can be safely predicted. In Amphibia and fresh-water fish, where secretion by the tubule may be small, special conditions must be employed to demonstrate it. On the other hand, tubular elimination or secretion is easily proven in marine teleosts (Marshall, 1930). Just how much secretion by the tubule will take place in a kidney would appear, then, to depend on

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[5] Of course, some reabsorption of water and concentration of urinary constituents takes place in the tubule of the lower vertebrates.

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the amount of filtrate elaborated by the glomeruli and hence on glomerular development and activity.

SUMMARY

Evidence is presented for the view that the glomerular development of the kidneys of vertebrates is related to water excretion. The protovertebrate kidney was at one stage probably aglomerular and the glomerulus was evolved as an adaptation to a fresh-water habitat. In the lower vertebrates remaining in fresh water (dipnoans, ganoids and fresh-water teleosts) and in those still in intimate dependence on it (Amphibia), the glomerular development is good; but with the secondary assumption of a marine habitat (marine teleosts) or with the assumption of terrestrial life in which water conservation becomes a necessity (arid-living reptiles and birds) the glomerular development is extremely poor. In the mammals (and possibly to some extent in lower vertebrates) the primitive water-excreting function of the glomerulus has been secondarily diverted to a filtration-reabsorption system designed to excrete waste products without the loss from the body of excessive quantities of water. The relative importance of tubular secretion in any kidney will, on this view, depend upon the extent of glomerular development.

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PLATE I

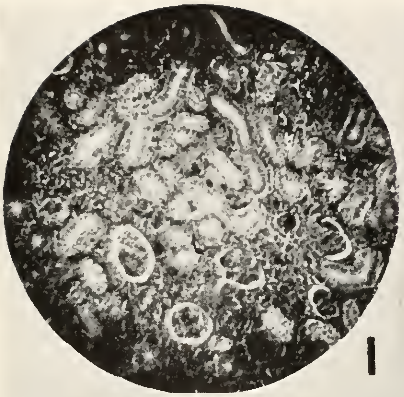
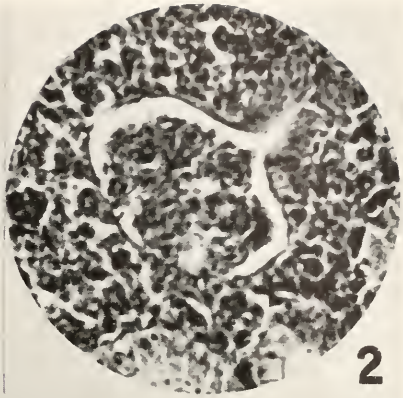
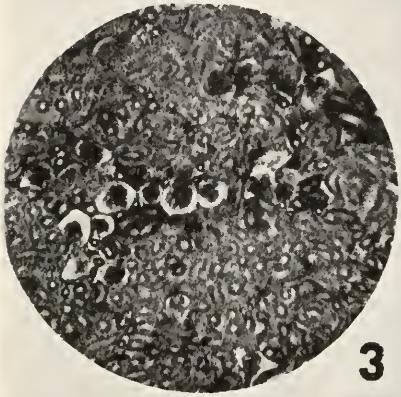
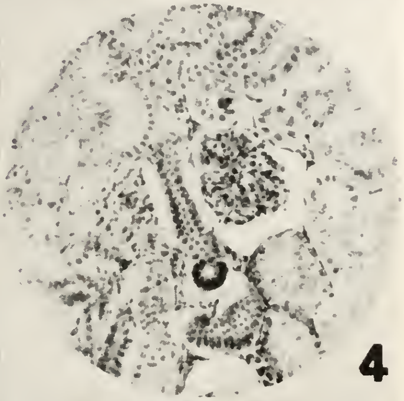
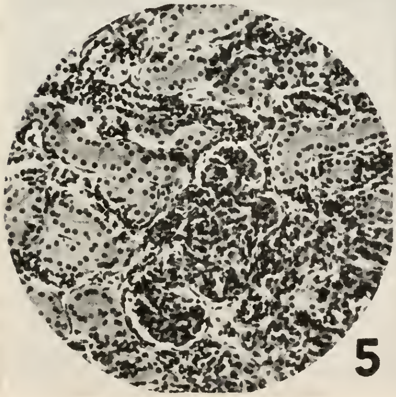
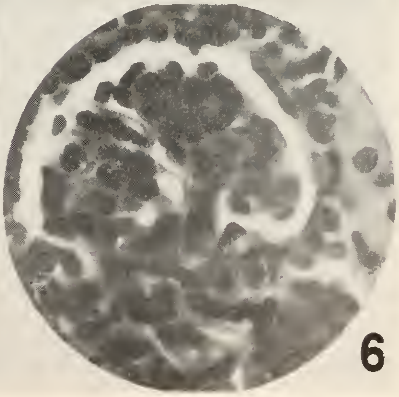
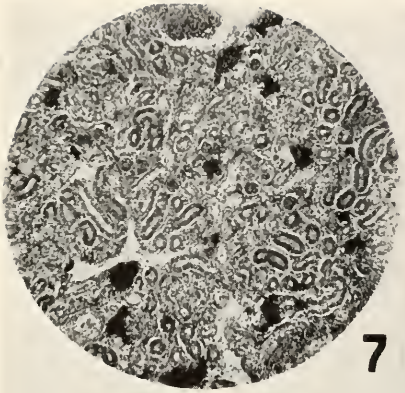







FIG. 1. Section of kidney of Cat fish (*Ameriurus nebulosus*), group I. × 63.
FIG. 2. Higher power of portion of field shown in Fig. 1. × 262.
FIG. 3. Section of kidney of Silver perch (*Morone americana*), group I. × 63.
FIG. 4. Higher power of portion of field shown in Fig. 3. × 262.
FIG. 5. Section of kidney of Lung-fish (*Protopterus aethiopicus*), group I. × 63.
FIG. 6. Higher power of portion of field shown in Fig. 5. × 262.

PLATE II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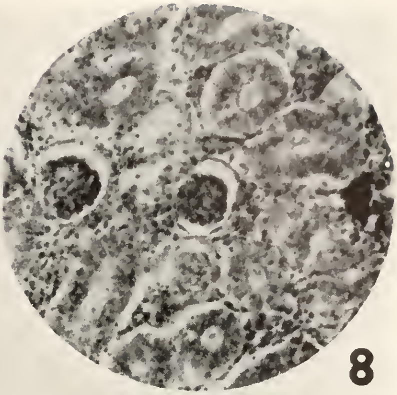
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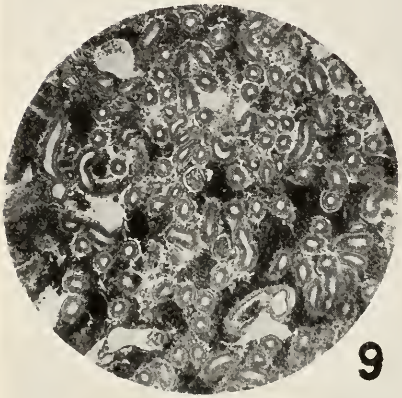
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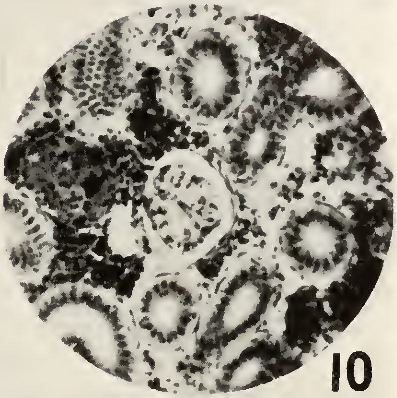
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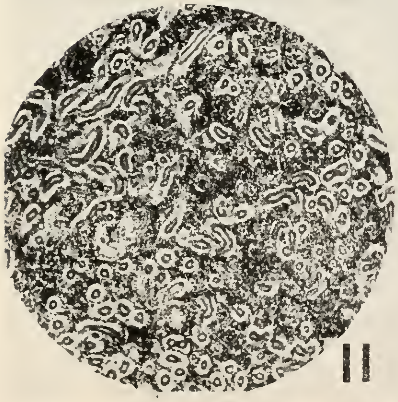
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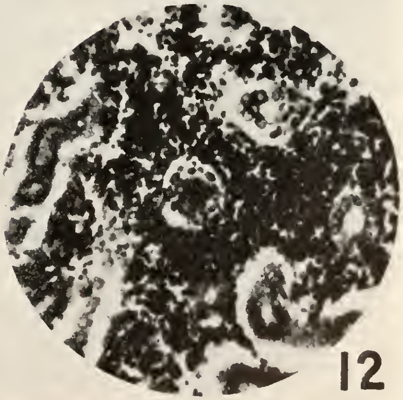
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FIG. 7. Section of kidney of Jack (*Caranx ruber*), group II. × 63.

FIG. 8. Higher power of portion of field shown in Fig. 7. × 262.

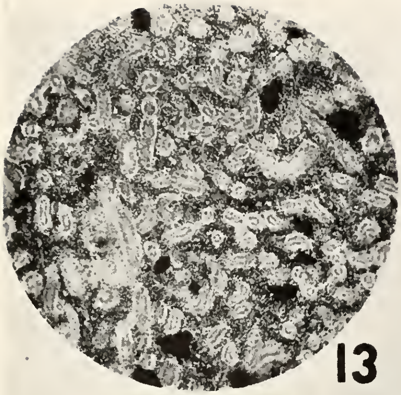
FIG. 9. Section of kidney of Flounder (*Pseudopleuronectes americanus*), group II. × 63.

FIG. 10. Higher power of portion of field shown in Fig. 9. × 262.

FIG. 11. Section of kidney of Brown tang (*Teuthis hepatus*), group III. × 63.

FIG. 12. Higher power of portion of field shown in Fig. 11. × 262.

Plate III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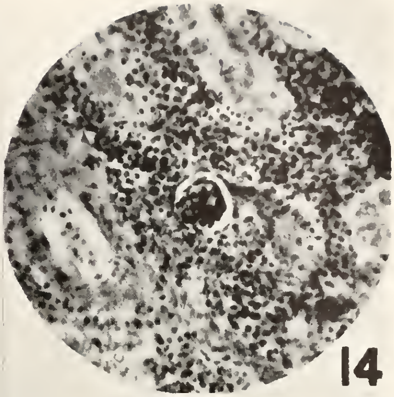
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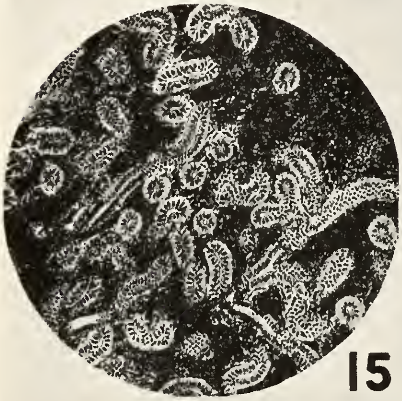
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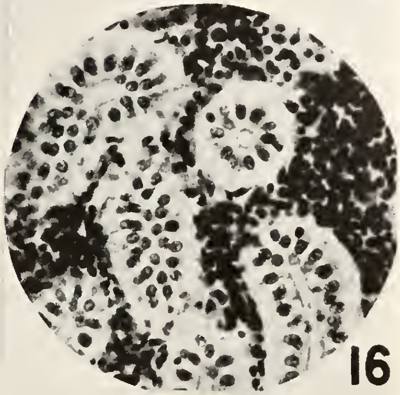
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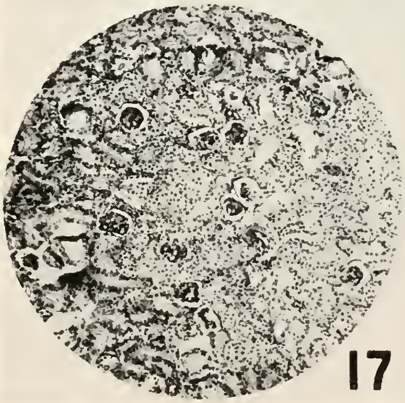
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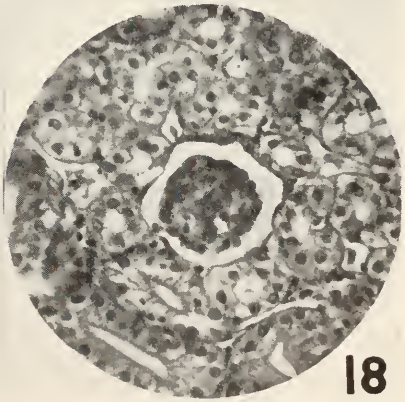
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Fig. 13. Section of kidney of Shellfish (*Lactophrys bicaudalis*), group III. × 63.

Fig. 14. Higher power of portion of field shown in Fig. 13. × 262.

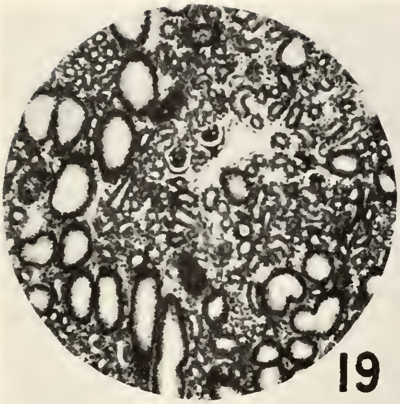
Fig. 15. Section of kidney of Toad fish (*Opsanus tau*), group IV. × 63.

Fig. 16. Higher power of portion of field shown in Fig. 15. × 262.

Fig. 17. Section of kidney of Terrapin (*Pseudemys sp.*). × 63.

Fig. 18. Higher power of portion of field shown in Fig. 17. × 262.

PLATE IV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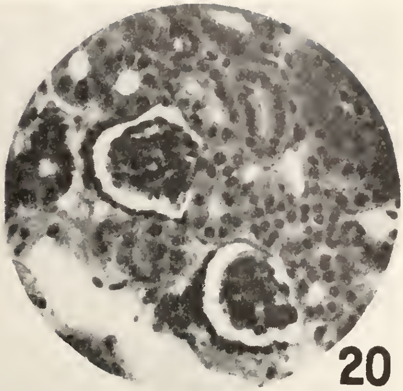
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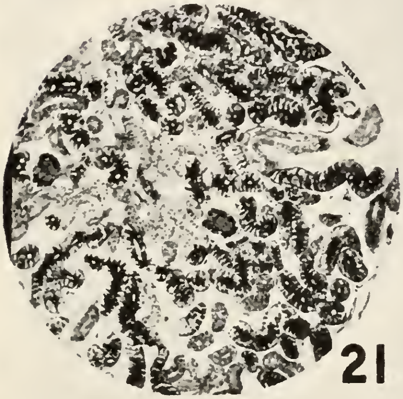
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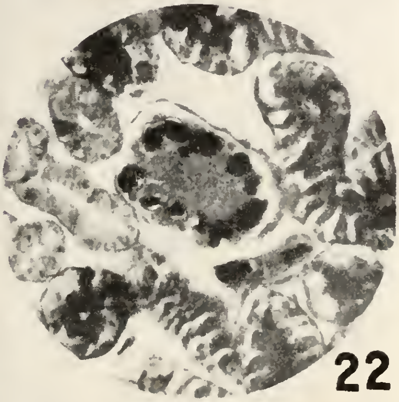
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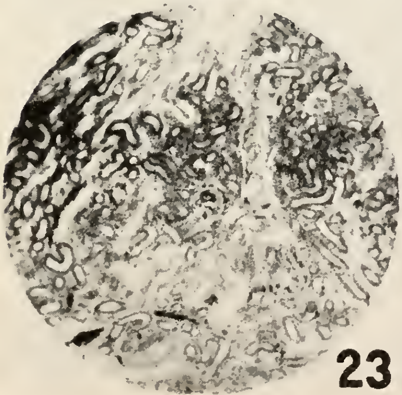
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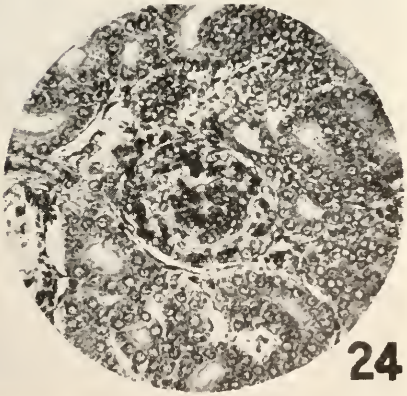
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FIG. 19. Section of kidney of Horned toad (*Phrynosoma cornutum*). × 63.
FIG. 20. Higher power of portion of field shown in Fig. 19. × 262.
FIG. 21. Section of kidney of Boa constrictor (*Boa imperator*). × 63.
FIG. 22. Higher power of portion of field shown in Fig. 21. × 262.
FIG. 23. Section of kidney of chicken. (*Gallus domestica*). × 63.
FIG. 24. Higher power of portion of field shown in Fig. 23. × 262.

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THE FORMATION, REGENERATION, AND TRANSPLANTATION OF EYES IN PECTEN
(*GIBBUS BOREALIS*)

EARL O. BUTCHER

(*From the Biological Laboratory, Hamilton College, Clinton, New York and the Marine Biological Laboratory, Woods Hole, Mass.*)

INTRODUCTION

One of the most highly organized and fascinating eyes among the invertebrates is found in *Pecten*, the common scallop. Especially good descriptions of the histology of the eye, which occurs along the mantle edge in the various species, may be found in the articles of Patten (1887), Hesse (1900), Hyde (1903), and Dahlgren and Kepner (1908). Drew (1906) also has a review of the structure in his excellent work on the giant scallop.

It was originally planned to find out if the mantle edge could be stimulated in any way to form eyes, or, in other words, to find the determining factor of these eyes. Before any information could be collected on the proposed experiment, various problems were confronted which necessitated a preliminary study. Among these problems were the following questions: (1) Do more eyes form as the individual grows and more space is provided for them? (2) How do the eyes develop? (3) Will the eyes regenerate? (4) Does the nervous system exert any qualitative or quantitative influence on the regeneration of the eyes? (5) Will the eyes grow and differentiate when transplanted to places other than the edge of the mantle?

These and innumerable other questions are of such interest that the results of the findings seem to warrant reporting.[1]

THE STRUCTURE AND THE FORMATION OF THE EYES

The eyes of *Pecten*, metallic in appearance, are scattered along the mantle edge. They are located on short stalks interior to numerous tentacles which are adjacent to the shell. The tentacles overhang the eyes in such a manner as to provide a shade for them. Sometimes the eyes are placed at almost equal distances from one another, but,

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[1] The writer wishes to acknowledge, at this time, his indebtedness to Dr. H. B. Adelmann of Cornell University for suggesting this study.

for the most part, their arrangement is of an irregular nature. More eyes are always found on the left or upper mantle.

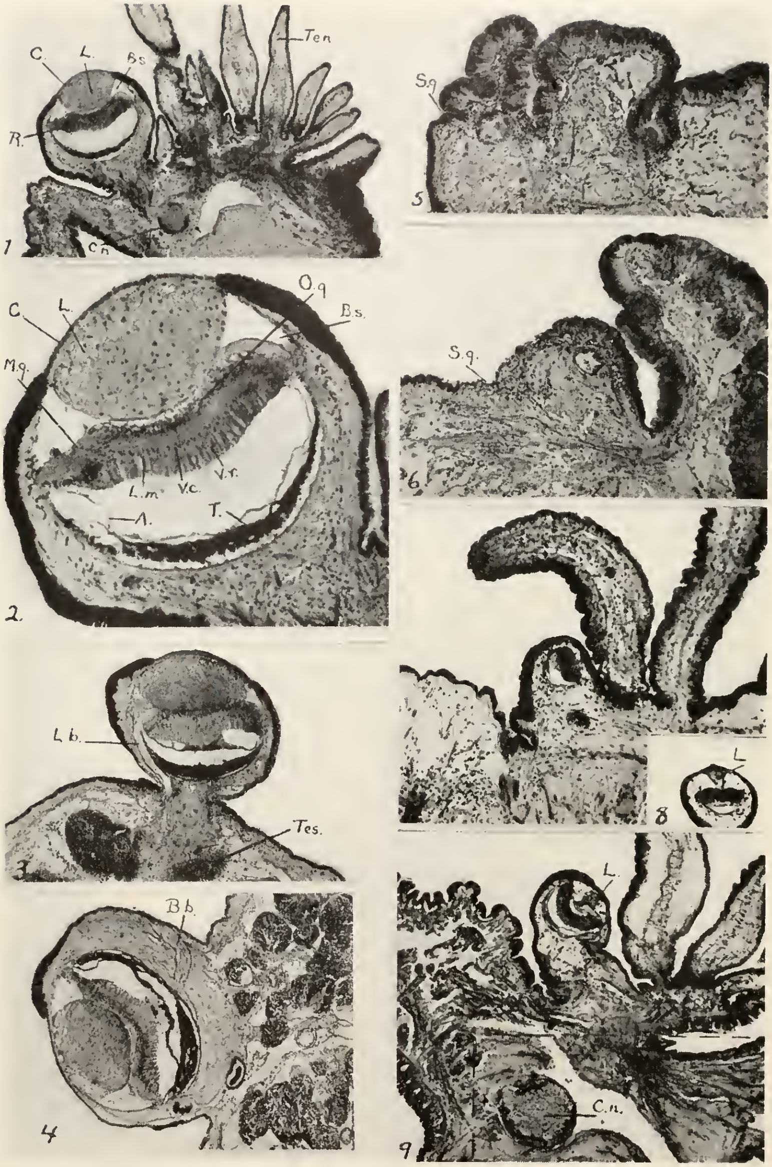
The eye of *P. gibbus borealis* consists of an epithelial covering which is modified into a cornea. Inside this covering we find a cellular lens, a blood sinus, the several layers of the retina, a space filled with vitreous humor, the argentea, and lastly the pigmented tapetum (Figs. 1 and 2).

The lens, oval in shape, is separated from the cornea by a thin layer of connective tissue. Its edges reach as far as the pigmented area of the cornea or iris. Its proximal curve, projecting into the blood space, almost touches the retina. Structurally, the lens consists of cells which contain peripherally located nuclei and a vacuolated cytoplasm.

The retina extends across the eye sac between the anterior blood space and the posterior vitreous humor. The thick layer of visual cells are found on the proximal surface of the retina. Their distal ends are in the form of long, thick, visual rods. The place where the visual cells are continued as the rods is straight and even and is marked by a set of fine plates in the substance between the cells. These plates constitute the limiting membrane (Fig. 2). Miss Hyde thinks that each rod in *Pecten irridans* has a nucleus and is separate from the visual cell. Dahlgren and Kepner did not find these nuclei in the rods of *Pecten tenuicostus*, and my preparations, although stained in iron hæmatoxylin, do not show separate components as described by Miss Hyde. Since the visual cells are on the proximal surface, the retina may be considered as inverted. In this respect, it resembles the vertebrate eye.

Lying distal to the visual cells of the retina is a layer of cells which constitute the outer ganglionic cell-layer. Dahlgren and Kepner find that nerve and supporting cells constitute this layer. According to them, neurofibrils enter it from the lateral branch of the optic nerve, pass through the cytoplasm of the nerve cells, and then ramify inwardly to send branching ends to the visual units. The visual cells also establish direct connection with large ganglionic cells along the margin of the retina (Fig. 2).

The optic nerve approaches the eye and divides into two branches, the basal and the lateral. The basal branch (Fig. 4) was thought by Miss Hyde to be connected with the marginal ganglionic cells, while the lateral division (Fig. 3) was related to the external ganglionic cell-layer. The basal branch was also considered by Miss Hyde to be afferent, while the efferent path coursed over the side branch to the external ganglionic cell-layer.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One finds a variation in the number of eyes between individuals of the same size. More eyes are found in large than in small *Pecten.* The question at once arises whether or not new eyes form or whether they are always present yet in somewhat of a latent condition. Patten described briefly the embryology in 3 mm. *Pecten.* However, he did not investigate the problem just presented. Apparently, new eyes are not added in accordance with any particular plan, but make their appearance as space for them is provided.

The occurrence of eye formation in the middle of the shell is only occasional. Near the ears of the shell, one is more apt to find stages in development, showing that most of the growth must occur in this region. Stages of formation, of course, are more frequently found in smaller than in larger individuals.

At the inner edge of the tentacles one finds a shallow groove which may be called the ophthalmic groove (Figs. 5 and 6). It is at the base of this groove that formation begins. The first indication of an eye

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FIG. 1. Transection of the mantle edge to show the position and structure of a large eye. × 50.

FIG. 2. A magnified section to show the detailed structure of an eye. × 140.

FIG. 3. This photograph shows an eye which has developed in the gonad. It was transplanted at about the same stage as the one illustrated in Fig. 7. Note the lateral branch of the optic nerve which had grown about an inch into the gonad. × 80.

FIG. 4. This eye was transplanted to the gonad when about the size of the one illustrated in Fig. 1. Note that it has continued to enlarge and that the basal branch of the optic nerve has grown considerably. × 60.

FIG. 5. This photograph shows a stage in the development of an eye. The vesicle has differentiated from the downgrowth which occurred at the point *S.g.* × 95.

FIG. 6. A slightly later stage in the development of an eye. Note that the right side is thickening to form the retina, while the other side will thin to form the posterior part of the eye. × 95.

FIG. 7. This figure shows the retina and its development more distinctly. × 95.

FIG. 8. This section shows the beginning of the lens formation from connective tissue cells above the retina. × 95.

FIG. 9. Further differentiation of the eye is illustrated. Note also that the eye has been pushed outward as a result of the lengthening and rotation of its stalk. × 95.

The left side of Figs. 1, 2, 5, 6, 7, and 9 represent the inner side or toward the light while the right side of these figures is next to the shell or the outer side.

ABBREVIATIONS

A., argentea
B.b., basal branch of optic nerve
B.s., blood sinus
C., cornea
C.n., circumpallial nerve
L., lens
L.b., lateral branch of optic nerve
L.m., limiting membrane
M.g., marginal ganglionic cells
O.g., outer ganglionic cell-layer
R., retina
S.g., ophthalmic groove
T., tapetum
Tes., testis
V.c., visual cells
V.r., visual rods

is a downgrowth. These downgrowths may be hollow invaginations or nearly solid buds. The whole papilla then elongates, and gradually loses its connection with the surface epithelium (Fig. 5). Meanwhile, it has begun its transformation into an optic vesicle, the cells of which are distinctly provided with cell walls. Some cells are greatly pigmented, although pigmentation may be entirely lacking (Fig. 6).

An increase in the number of cells by mitosis soon occurs in the side of the vesicle which is farthest from the light (Fig. 7). The cells continue to increase in number on this side until several layers are present. Meanwhile, differentiation begins and the different layers of the retina appear. Those cells more central (with respect to vesicle) aline themselves as the visual cells, while those more peripheral form the ganglionic cell-layer (Figs. 8 and 9).

The single layer of cells, which forms the wall of the vesicle next to the light (Figs. 7 and 9), is first filled with colorless granules which gradually acquire a red color. These granules are located particularly in the periphery of the cell. Apparently the argentea is derived from the clear refractive distal or innermost part of these cells. Later it separates from the more pigmented peripheral portion, and becomes compressed into a thin layer which is devoid of pigment. The pigmented part persists as the definitive tapetum. Within the vesicle arises the vitreous network, a homogeneous fluid that has a vacuolated appearance.

As the retina continues to differentiate, it becomes concave (Fig. 9). Connective tissue intimately surrounds the optic cup. In the region of the concavity of the retina the connective tissue cells become pigmented and spherical. As the cells aggregate together, the nuclei become peripherally located, and gradually the lens appears (Figs. 8 and 9).

The eye begins its formation at the base of a groove, but with further development, it is pushed outward at the end of a stalk. The retina starts to develop turned away from the light, but as the stalk lengthens, it is rotated toward the light. With increase in size the eye occupies the entire end of the stalk.

The epithelium directly above the lens loses its pigmentation and forms the cornea. That at the edges of the lens, however, does the opposite, or becomes highly pigmented to form the iris. Nerves grow out from the ganglionic cells of the marginal and external ganglionic cell-layers in the direction of the circumpallial nerve.

Abnormalities in development frequently occur. Large pigmented undifferentiated vesicles are commonly encountered. Apparently the stimulus has been present and has caused the vesicle, but, for some reason, there has been no further differentiation. One also finds eyes completely upside down, especially on short stalks near the ear of the shell. Externally, such an eye gives one the impression that the entire cornea is pigmented, but upon sectioning it one observes that it was the pigment layer of the eye which was outward. This is, no doubt, due to the fact that the stalk containing the eye did not lengthen sufficiently to allow for a rotation.

Duplication is, likewise, commonly encountered. Oftentimes a retina will have two concavities, each having a small lens. Two eyes, and even three are occasionally found on the same stalk. Observations show that such a condition resulted from the forking of the downgrowth in the beginning stage of development. The factor or factors which are influential in causing this condition have not been found.

Many irregularities are manifested in the rate of development. The optic cup may be nicely differentiated and the lens may be entirely lacking. The opposite extreme, or the early formation of the lens in the presence of small undifferentiated retina, also occurs, although not frequently.

A thorough search has given no insight on the stimulating factor in the formation of an eye. Pigmentation, nerve endings, attachment of muscles have all been carefully considered, yet these fail to give satisfactory reasons for the presence of the eye. The question regarding the determination of the eye will be considered more fully when discussing the regeneration of the eye.

The potentiality for forming eyes appears to be gradually lost as the *Pecten* become larger. The number in large individuals seems to be fairly constant.

The Influence of the Nervous System on the Regeneration

Before going further, it might be appropriate to outline briefly the plan of the nervous system of *Pecten*. At the sides of the gullet there are two distinct and separate cerebral ganglia which are united by a cerebral commissure. Each ganglion gives off a cord, the cerebropedal connective, which passes downwards and backwards to the pedal ganglion. From each cerebral ganglion there also proceeds directly backwards a long cerebro-visceral connective, which ends in a visceral ganglion placed on the ventral side of the adductor muscle. The visceral ganglia of the two sides are fused to each other by a ganglionic commissure. In the edge of the mantle or pallium, we find a nerve, the circumpallial nerve (Figs. 1 and 9), which contains some nerve cells. This nerve is connected with the visceral ganglion by a number of nerves, the posterior pallial nerves, while anteriorly it receives a supply from the cerebral ganglion, the anterior pallial nerves.

Pertaining to the problem of regeneration, we are confronted with the following questions: (1) Will the eyes reappear upon removal? (2) Does the nervous system exert any influence on this reappearance? (3) What factors are responsible for the reappearance of the eyes?

One has to refer only to the literature to learn of the amazing number of articles pertinent to the subject of the influence of the nervous system on regeneration. Herbst (1916) found that the eyes of certain Crustacea regenerate normally only when the optic ganglion of the animal in question is not injured. When the optic ganglion was removed together with the eyes, heteromorphic bodies, having the strucure of feelers, were produced instead of eyes.

In Mollusca, the studies of König (1915) and Nonne (1925) on the gastropods is interesting. König found that in *Arion empiricorum* the regeneration of the eyes took place independent of the tentacle ganglion, cerebral ganglion, and nerves. More recently, Nonne observed that the eye of *Tachea hortensis* would regenerate if the corresponding cerebral ganglion were absent. He found that it took longer for the eye to regenerate on the injured side than it did on the normal side. Likewise, the eye often remained smaller. He concluded, therefore, that the nervous system had a quantitative influence on the eye regeneration rather than a qualitative. It was thought that it might be interesting to see if similar conditions existed in the other Mollusca, particularly the lamellibranchs (Pelecypoda) with which this investigation is concerned.

Normally, *Pecten gibbus* rests on its right side, which is usually somewhat lighter and less pigmented. One will find along the edge of the right pallium of a 3.5 cm. *Pecten* an average of about thirty-five eyes, while on the left or upper mantle forty-five is about the average number.[2] This size (3.5 cm.) usually has as many eyes as the larger *Pecten*, and, since they were more easily handled than larger ones, they were more frequently used for experimentation. However, much smaller and much longer *Pecten* were employed.

If one removes the eyes from one of the mantles of a 3.5 cm. *Pecten*, a similar number will reappear in about forty days. In smaller *Pecten* they reappear sooner, and in larger *Pecten* they reappear more slowly. Both sides regenerate in a similar manner when the eyes are removed from both mantles. These experiments establish rather definitely the fact that the eyes would reappear upon removal. Sections of the mantle showed that the regeneration took the form of ordinary development which has previously been described.

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[2] This measurement was recorded at the hinge.

Several possibilities existed wherein one might learn whether or not the nervous system exerted any influence on the reappearance of the eyes. One might remove part of the visceral ganglion that supplies pallial nerves to the circumpallial nerve. The pallial nerves and circumpallial nerve could also be cut.

In the cases where the visceral ganglion was removed from one side and the eyes from both sides, the eyes reappeared on both sides equally as fast. When compared to cases where only the eyes were removed, no difference was found in the time interval that it took for the eyes to form again. Removal of the eyes from both sides and the cutting of the pallial nerves from one side had no effect on the reappearance, for a similar number of eyes was found on both mantles after twenty days. Even cutting the pallial and circumpallial nerve on one side and removing the eyes from both mantles had no effect on the rate of appearance between the two sides. It was, likewise, found that it made no difference in the time necessary for reappearance if the eye was removed or the entire eye and stalk. As usual, a new eye formed by a downpocketing at the base of the groove.

These experiments might be subject to criticism since the circumpallial nerve contains ganglionic cells within itself and this had not been removed. In order not to leave this questionable, the eyes were removed from the entire mantle and about five-tenths cm. of the length of the mantle, excluding the ophthalmic groove, was cut away. The dotted line in Fig. 9 illustrates approximately the section that was taken away. The material removed was later sectioned to confirm that the circumpallial nerve had been extracted. Thirty-four days later the *Pecten* were examined and many eyes had reappeared on the mantle where the nerve had not been removed. Where the nerve had been cut away, the eyes were very small and in the first stages of development. They were also less numerous. Since the eyes were less numerous and less developed, the conclusion seemed justified that the nervous system did exert a slight quantitative influence on the reappearance. Disturbance of vascular relations could, however, have accounted for a portion of this extra time that it took for the eyes to reappear.

From all of the various experiments, one could, at least, conclude that the nervous system had little influence on the regeneration and development of the eyes and that the eyes were self-differentiating.

TRANSPLANTATION OF EYES

In order to test the self-differentiating property of the eyes further, it was decided to transplant anlagen and fully developed eyes to places remote from their original location. The edge of the gonad was found to be a very desirable place to anchor and to grow the eyes.

The earliest identifiable stages (Fig. 7) were transferred to the edge of the gonad and, after an interval of three or four weeks, the gonads were removed and the eyes were found to have developed normally in every respect (Fig. 3). The nerve, both lateral and basal branches, had made a considerable growth into the gonad. Progressive and normal differentiation had occurred in all parts of the eye.

Large eyes, when transplanted to the edge of the gonad, retain their normal condition and in no way show signs of regression. Instead the eyes enlarged (Fig. 4) and the optic nerve made a considerable growth into the gonad.

When the optic cup was planted without the lens, the invading connective tissue cells were stimulated to take characteristics of lens cells. Portions of the optic cup and of the lens, however, degenerate when planted alone.

DISCUSSION

It has previously been pointed out that Hyde thought that the retina of the eye was provided with two different branches of the optic nerve. The basal branch has been designated as afferent, while the efferent path coursed over the side branch. If the lateral branch were mainly efferent in nature, then one might think that the nerves constituting it would grow towards the eye. In the study of the embryology of the eye, I do not find that this occurs, but that the lateral branch grows away from the eye. This would indicate that the cell bodies of these fibres were in the retina. It appears that the external ganglionic layer of cells serves this purpose.

Further evidence that the cell bodies of the nerve fibres of the lateral branch are located in the retina is seen in transplanting the eyes. If these are entirely efferent fibres in the lateral branch, then one might expect them to degenerate or not develop when the eye is transplanted. This is not the case, however, as may be seen in Fig. 3. This photograph shows the lateral branch quite normal and making a growth into the gonad. Therefore, the lateral nerve ganglonic cells must be located in the retina. The fact that the ganglionic cells of the basal and lateral branches are located in the retina, does not give any insight as to their function. From observations, I believe that the lateral branch receives stimuli from the central part of the retina, while the basal branch gets its supply from the margin of the retina. Dahlgren and Kepner apparently think that there are two different pathways for the impulse.

As to the function of the eyes, we know little. Patten found that *Arca noae* was extremely sensitive to light. Shadows and small objects, within two or three inches, caused it to close at once. It has been suggested that the eyes of *Pecten* may help them to avoid enemies. They may also prevent them from being left on dry land in case of tide. The growing intensity of light, in this case, would seem to act as a stimulating agent.

Why we have more eyes on the left or upper mantle is interesting. Is it because more eyes are needed to see moving objects on the bottom, or is it because there is less light and more eyes are needed for acuity? Until something is learned about the determining factor of the eyes, it will be difficult to give a hypothesis for the occurrence of more eyes on the upper side of the mantle. Similarly, it is difficult to arrive at any definite conclusion for the orientation of the eye in development. Why does it always develop away from the light?

Equally of interest and inexplainable is the presence of large undifferentiated vesicles. In this case the stimulus was apparently present at first and then subsided, or did the tissues have eye potentiality and then lose it?

As to the nature of the determining factor of the eyes, I am unable to state at present. Pigmentation, nerve endings, attachment of muscles, light, and injury are all suggested.

The epithelium covering the tentacles contains a variable amount of pigment. It was thought that the amount of pigment might in some way stimulate development. However, the downgrowths had about an average amount of pigment, and as far as I was able to determine, eye development occurred whether or not there was an abundance of pigment or a lack of it.

There was no evidence that any nerve had come in contact with the epithelium and had acted as a stimulating factor in producing a downgrowth. Even the eye vesicle had formed previous to the insertion of the retractor muscle into the edges of the cornea, and the muscles, or their attachment, therefore, seemed to have no part in the formation of the eye.

Whether or not light may in some way stimulate the epithelium is still to be investigated. It would appear that it did not, since the eyes develop away from the light.

It would seem that the only possible solution left was that the epithelium alone possesses the potentiality for forming eyes. This

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power must be evenly distributed, since eyes appear almost anywhere. It would be interesting to transplant ophthalmic groove to gonad, to invert mantle edge, to transplant other epithelia to edge of the mantle in order to learn something about the determining factor of the anlagen of the eyes.

Even if this property of the epithelium for forming eyes is rather distributed, there must be some stimulus that evokes eye development.

CONCLUSIONS

1. Eyes continually form in *Pecten* as the individual grows and more space is provided for them.
2. Eyes will reappear upon removal, and the nervous system exerts very little influence on the reappearance.
3. Eyes and eye anlagen can be transplanted to other parts of the body. Transplanted eyes grow and retain a normal condition, and the optic nerve attempts to establish normal relations.

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REVERSAL OF PHOTOTROPISM IN A PARASITIC WATER MITE

JOHN H. WELSH

Zoölogical Laboratory, Harvard University

Reversal in the phototropic response of certain animals sometimes occurs in the course of normal development and has been brought about in the laboratory by mechanical stimulation, change in temperature, change in light intensity, starvation, and chemical stimulation. In many cases the reversals produced artificially are apparently non-adaptive, or at least of no evident use to the organism. While working with certain parasitic water mites it was found that material from the host causes a reversal in the light response which is evidently of an adaptive nature. The reversal was studied in normal animals in directional light and also in animals blinded in one eye. The destruction of one eye brings about circus movements which are reversed by an extract of materials from the host.

The mite used in the study was *Unionicola ypsilophorus* var. *haldemani* (Piers). It lives between the inner and outer gills of the common fresh water clam or mussel, *Anodonta cataracta* Say, and is commonly called a parasite although it may be only commensal with the clam. The number of mites found in an individual clam varied from one to fifteen, the average for a large number of clams being 5.3 mites. Only very rarely were clams found without some mites present. According to Wolcott (1898) these mites seldom leave the host. However, they must do so at times, otherwise young clams would not become infested. During the course of the experiments specimens of *Anodonta* were kept under various conditions of light, temperature, and oxygen tension but on only two occasions were mites known to leave the clams. They seem to feed largely on the mucus from the gills and their presence in large numbers appears to increase the amount of mucus secreted.

When removed from the gills and allowed to remain in water from the mantle cavity, the mites are indifferent to directional light, wander aimlessly about the dish, and tend to cling to bits of mucus and gill. If washed and placed in fresh water free of material from the host they show a definite positive phototropism. Among a large number of individuals a few may remain indifferent to light but the majority show a strong photopositive reaction an hour or so after removal from the host.

When a number of mites are placed in an aquarium with a clam they soon find their way inside the clam. In one case forty-five mites were placed in a small aquarium with a single clam. The following day only two could be found free in the aquarium. The clam was opened and forty-eight mites were removed from between the gills, all but five of these having gained entrance to the clam over night.

The problem presented by the behaviour of the mites was to explain why they remain within the host although photopositive after removal, and how they are attracted to the host when free in an aquarium.

When a number of mites are placed in water containing pieces of gill they soon locate the gill, particularly if in the dark or in diffuse light. They tend to crawl into the gill by way of the cut edges or to collect under the pieces, there remaining quiet. This would seem to indicate a positive stereotropism. It is believed that a definite chemical stimulus directs the mites to the material from the host and that this attraction aided by a positive stereotropism keeps the mites within the clam even when a strong beam of light is projected into the mantle cavity.

During the early observations on the behaviour of these mites another interesting phenomenon was noted. Mites which were distinctly positive to light exhibited an immediate reversal to a photonegative state when transferred to a water extract of clam gill or to water from the mantle cavity of the clam. This reversal was at times only temporary but might last for a period of an hour or more. It was more pronounced and lasted longer if the mites had been out of the host for two or three days.

An experiment to show the effect of dilution of gill extract on the reversal of the light response illustrates the usual type of reaction.

Four pairs of gills were ground in a mortar with 20 cc. of distilled water. The mixture was centrifuged, the liquid decanted and filtered. 5 cc. of this extract, which for convenience may be called a 100 per cent solution, were placed in a small rectangular glass jar, 8 cm. long and 2 cm. wide. This was illuminated from one end with a beam of light from a 25 watt lamp at a distance of 15 cm., projected through an opening 2 cm. in diameter in an opaque screen. The light was reduced in intensity by interposing a sheet of tracing paper between the lamp and the jar. The end of the jar away from the light was covered on the inside with dull black paper coated with paraffin in order to prevent reflection of light. Ten photopositive mites were placed in the end of the jar nearest the source of illumination and their reactions noted.

For each dilution of extract a fresh lot of mites was used in order to avoid an accumulative effect.

Following are the results of using varying strengths of the original extract from 100 to 0.01 per cent:

100 per cent	10 mites immediately negative
50 per cent	9 mites immediately negative
25 per cent	8 mites immediately negative
10 per cent	8 mites immediately negative
5 per cent	5 mites immediately negative
2.5 per cent	6 mites negative (an obvious increase in the reaction time before the mites begin to move away from the light).
1.0 per cent	4 mites negative
0.05 per cent	2 mites negative
0.025 per cent	2 mites negative
0.01 per cent	0 mites negative

Only those mites which moved away from the lighted end of the jar and remained negative to light for an appreciable length of time were considered to have undergone a reversal in their response to light.

In control experiments handling and mechanical disturbance as well as the addition of small amounts of CO_2 were found to have no effect on the light response.

The reversal of circus movements of the mites confirmed the results obtained with directional illumination and offers a more striking demonstration of a change from a photopositive to a photonegative condition in an organism. Mites were blinded in the right eye by touching it with the point of a hot needle. The constant movement in circles toward the left or normal eye was sufficient indication that the blinding of the right eye had been successful. These animals showed remarkably uniform movements, travelling slowly, on ground glass, in circles with a diameter of 0.5 cm. When an extract of gill was added to the water near the mites, or when they were transferred to an extract of gill, an immediate reversal in the direction of circling would usually take place and the movement would be toward the blinded eye, indicating a change from a positive to a negative condition. Occasionally there would be failure to reverse or instead of complete reversal the movements would be at random.

An attempt was made to determine the nature of the substance in the gills which brings about the reversal in the light response and also exerts an attraction on the mites. Putrefaction at a temperature of 37.5° C. for a week was found to have no effect. At the end of this time the extract was as effective as fresh extract in causing a reversal.

Boiling for five to ten minutes likewise had little or no effect on the material which acts upon the mites. It is possible that decomposition products of the mucus or other proteins of the gills, which would not be affected by boiling or putrefying, are responsible for the reversal of the light response.

The organs concerned with the reception of the stimuli were likewise not determined. The removal of the labial palps at their basal joints in a number of mites had no apparent effect on the reversal or in the ability of these mites to locate and enter clams when free in an aquarium. As little is known concerning the chemical receptors in spiders and mites, it is difficult to say what centers are involved in the reversal.

DISCUSSION

The behaviour of the variety of *Unionicola* which was studied seems to indicate a change from a primitive photopositive to a photonegative condition, due to a parasitic or commensal life. Negative phototropism assisted by chemotropism and stereotropism keeps the mites within the host. Upon removal from the influence of the clam, or material extracted from the clam, the mites revert to a photopositive state.

The rapid reversal from a photopositive to a photonegative condition when stimulated by extract of gill, or by water from the mantle cavity, is probably a central nervous phenomenon, and might be called a " conditioned reflex." Doubtless many parasitic animals become conditioned to stimuli from the host and show adaptive reactions quite different from their primitive responses.

Arey and Crozier (1921) investigated a case of change from independence of heliotropism to negative phototropism in *Onchidium,* a pulmonate mollusc, and their results are summed up in the following statement: " When tested apart from their specific normal environment the *Onchidia* are always negatively phototropic. In the natural state their movements are entirely independent of heliotropism . . . the simultaneous return to the nest on the part of the various members of a colony can be understood on the assumption of a ' reversal of inhibition ' brought about by substances derived from materials while feeding." This case, while not identical with that described above, does indicate an effect of some substance in the food or in the environment on the light response of an animal.

Cole (1922–23) investigated the light response of *Limulus* and found that it might be modified by (*a*) fright, (*b*) hunger, (*c*) stereotropism, (*d*) photokinesis, and (*e*) unknown stimuli. He states: " It becomes clear after observing *Limuli,* however, that they are fundamentally and primitively positive to light, but that many factors may modify or mask the phototropic reaction."

Stier (1926) studied a case of reversal of phototropism in *Diemyctylus,* the spotted newt, and found that negative phototropism is characteristic of animals from which food has been withheld, while feeding reverses the response.

SUMMARY

1. The water mite *Unionicola ypsilophorus var. haldemani* (Piers), living between the gills of the fresh water clam *Anodonta cataracta* Say, shows a positive reaction to light after removal from the clam.

2. An extract of the gills, or water from the mantle cavity of a clam, will cause a reversal to a photonegative state.

3. This reversal may be considered adaptive, for, aided by a positive chemotropism and stereotropism, it enables the mites to enter and remain within the host.

4. The phenomenon due to the parasitic life may be described as a " conditioned reflex "; the positive response to light being primitive and the negative response more recently acquired.

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REFLEX CARDIAC AND RESPIRATORY INHIBITION IN THE ELASMOBRANCH, *SCYLLIUM CANICULA*

BRENTON R. LUTZ

(*From the Zoölogical Station, Naples and the Physiological Laboratory of Boston University School of Medicine*)

In the higher vertebrates the effect of stimulation of sensory nerves on the medullary centers is rather complex since either acceleration or inhibition of the heart may be obtained, often associated with acceleration or inhibition of respiration. In mammals Brodie and Russell (1900) found that stimulation of the pulmonary afferent fibers is more effective in causing reflex inhibition of the heart than excitation of afferent cardiac fibers, and the visceral supply is least effective. Bainbridge (1920), working also on mammals, obtained no reflex cardio-inhibition from the afferent pulmonary fibers, but did get marked respiratory inhibition. He found no evidence that the cardio-inhibitory center and the respiratory center are associated except through blood pressure changes. Alterations in blood pressure in the medulla have sometimes obscured true reflex processes. Thus Anrep and Starling (1925) offered evidence to show that inhibition of the heart in mammals could not be obtained reflexly through afferent fibers from the heart and aorta, and pointed out that an increase in blood pressure in the medulla caused cardiac slowing. These results contradicted in part the conclusions of Eyster and Hooker (1908), who believed that not only increased blood pressure in the medulla caused cardio-inhibition, but that reflex slowing of the heart could arise from the aorta, with both afferent and efferent pathways in the vagus. In 1926, however, Anrep and Segall confirmed the latter authors. Heymans (1929) has found cardio-inhibition in dogs to arise reflexly from increased blood pressure in the carotid sinus.

A survey of the literature on fishes, both cartilaginous and bony, gives one the impression that there is a nervous association between the respiratory rate and the heart rate. Schoenlein and Willem (1894) said that cessation of respiration in *Scyllium canicula* and *Torpedo ocellata* caused a reflex from the pharyngeal cavity which inhibited the heart. Bethe (1903) and Baglioni (1907) have found respiratory and cardiac inhibition occurring together in selachians. Lyon, (1926) studying blood pressure and respiration in sand sharks (*Carcharias*), noted that a great variety of stimuli applied externally or to certain viscera caused inhibition of the heart and some sort of respiratory response. He pointed out an intimate relationship between the respiratory rate and the heart rate through the vagus.

Since neither vasomotor nerves nor accelerator fibers have been demonstrated in elasmobranchs (Schoenlein, 1895; Bottazzi, 1902; Müller and Liljestrand, 1918; Lutz, 1930*a*), one might expect to find in these fishes an association between cardiac and respiratory inhibition without the complicating blood pressure factors found in mammals. In elasmobranchs it is also possible, because of the persistence of the functions of the medullary centers without a blood supply, to investigate the influence of stimulation of sensory fibers from the heart without involving blood pressure changes.

Material and Method

Vigorous specimens of the dogfish, *Scyllium canicula,* averaging 300 grams, taken during the months of March and April from the Bay of Naples, were used. One specimen of *Scyllium catulus* (625 grams) was put through the usual procedures carried out on *S. canicula* and was found to respond in a similar fashion, but the smaller species was more easily handled and more abundant. The fish was removed from the water and the forebrain was separated from the rest of the brain by a transverse cut just in front of the optic lobes and destroyed. The spinal cord was transected at a desired level and pithed posteriorly. Transection at a high level, namely, between the fifth and sixth vertebrae or even more anterior, was found desirable, when the details of the experiment permitted, in order to keep the fish quiet. It was then placed ventral side up on a fish holder and the gills were perfused through the mouth. Respiratory movements soon became regular. The heart was exposed through the ventral wall of the pericardial chamber and a small gold hook was put through the superficial tissue at the tip of the ventricle. By means of a thread and a light writing lever its movements were recorded. A similar hook passed through the anterior border of the third or fourth gill slit was attached to a thread, passed horizontally to a pulley, and up to a light writing lever for recording respiration.

When necessary the vagus and its branches, including the beginning of the lateral line nerve and the last four branchial nerves, and the hypobranchial nerve were exposed quickly by cutting a slit with the scissors through the skin beginning above the fifth gill slit about 3 mm. ventral to the lateral line and running anteriorly toward a point just above the posterior angle of the eye. This opened into the fascia between the dorsal musculature and the gills and directly over the anterior cardinal sinus, which was slit lengthwise exposing the nerves on its floor. This involved some loss of blood, and care was taken to keep the fish in a horizontal position to prevent sucking air into the heart. A bloodless method, described by Hemmeter (1912), of exposing "the ramus cardiacus" was found not to be practicable on the 300 gram fish, especially since the heart of *Scyllium canicula* receives not only a branch from the visceral trunk of the vagus but also a branch from the fifth branchial nerve (4th branchial division of the vagus).

The first large sympathetic ganglion was exposed when necessary by opening the abdominal cavity. Since this ganglion lies within the posterior cardinal sinus, the latter must be opened with considerable unavoidable loss of blood.

RESULTS

Scyllium canicula was found to be very resistant to experimental procedures out of water when the gills were perfused. A preparation with the brain destroyed anterior to the optic lobes, the cord pithed posterior to the third vertebra, and the pericardial chamber opened, gave good cardiac and respiratory reflexes for over twenty-four hours. The laboratory temperature was about 14° C. When the anterior cardinal sinuses were opened, involving considerable bleeding, the preparation remained experimentally useful for about two hours. If in addition to the above procedures the abdominal cavity was entered and the posterior cardinal sinuses opened, medullary reflexes could be obtained for over an hour. In the latter case bleeding was profuse. When the aorta was transected, the respiratory center and the cardio-inhibitory center remained functional for over one hour (Fig. 1). Constant perfusion of the gills was found necessary to insure a quiet preparation, but without perfusion and with an intact circulation the

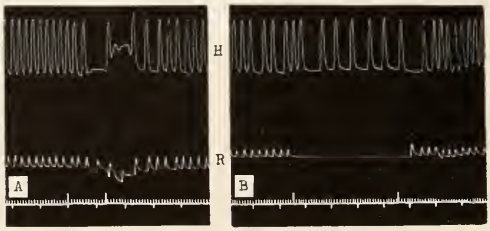

FIG. 1. Reflex cardiac and respiratory inhibition after transection of the ventral aorta. Forebrain and cord from the fifth vertebra destroyed. In this and subsequent figures the uppermost tracing is the heart record (*H*), the middle is respiration (*R*), and the lowest is the signal and time record. The large divisions are ten seconds. *A*, right fin pinched 21 minutes after transection of the aorta. *B*, gill perfusion stopped and started 31 minutes after transection.

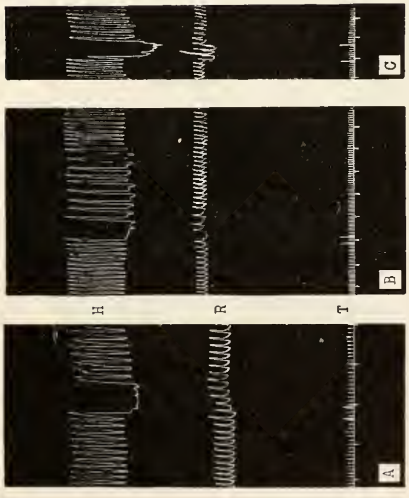

Fig. 2

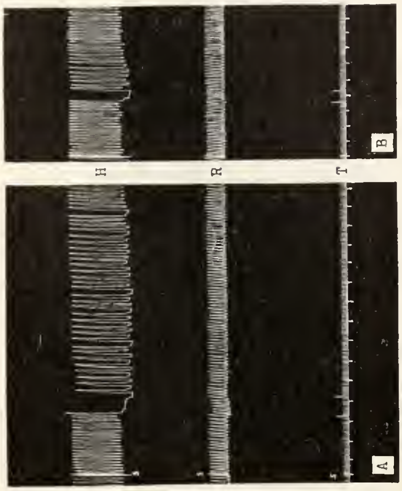

Fig. 3

Fig. 2. Cord pithed from the fifth vertebra. *A*, snout pinched gently with the fingers. Note reflex cardiac inhibition without respiratory inhibition. *B*, snout pinched vigorously. *C*, second right gill slit spread with forceps.

Fig. 3. Reflex cardiac inhibition without respiratory response. Cord pithed from the fifth vertebra. *A*, sides pinched with forceps dorsal to the pelvic fins. *B*, skin pinched on mid-abdomen.

medullary centers showed inhibitory reflexes for over three and one-half hours.

Cardiac and respiratory arrest or slowing were obtained with the cord pithed posteriorly from the second vertebra on mechanical and faradic stimulation of the skin of the head, nasal organs, gills, pectoral fins, cut surface of the coraco-mandibular muscle, pericardial walls, surface of the ventricle, oesophagus, stomach, spiral valve, and mesentery (Figs. 2, 3, 4 and 5). With the entire cord intact, similar responses could be obtained from the kidney, epididymis, ovary, and uterus. No response could be elicited from the liver or testis even with the entire cord intact.

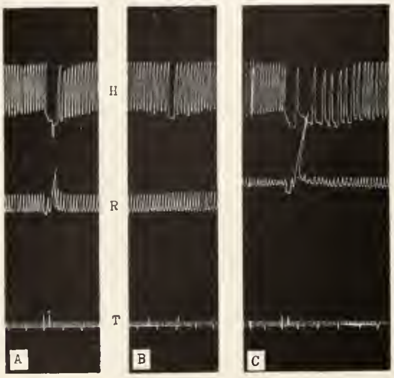

FIG. 4. Cord pithed from the fifth vertebra. *A*, needle stimulus to the left wall of the pericardial chamber. *B*, needle stimulus to the posterior wall. *C*, faradic stimulation of the posterior wall.

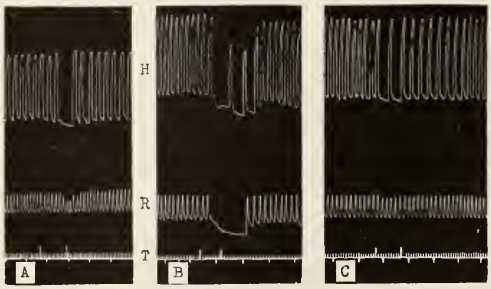

FIG. 5 Cord pithed from the third vertebra. *A*, stomach handled. *B*, posterior end of the stomach pinched with forceps. *C*, spiral valve pinched with forceps.

Faradic stimulation of the central end of the cut vagus, either the cardiac branch of the visceral ramus, the visceral ramus itself, or the cardiac branch of the fourth branchial division, the central end of the cut hypobranchial nerve, and the lateral line nerve (Fig. 6) produced cardiac and respiratory inhibition.

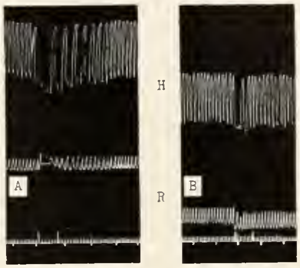

FIG. 6. Cord pithed from the eleventh vertebra. *A,* faradic stimulation of the central end of the left lateral line nerve. *B,* stimulus reduced.

Reflex cardiac inhibition and a reflex respiratory response were obtained when the rate of flow of the water to the gills was suddenly altered. The immediate response to starting and stopping the flow or to increasing and decreasing suddenly the rate was respiratory inhibition, usually followed by one or more rapid and vigorous contractions of the gill muscles. The heart generally, but not always, showed inhibition as well (Fig. 7). This response occurred also after the aorta had been cut to prevent any possible blood pressure effects through changes in water pressure on the gill vessels.

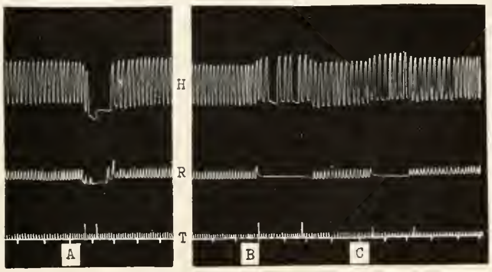

FIG. 7. Cord pithed from the fifth vertebra. *A,* water to the gills turned suddenly off and on. *B,* rate of flow of water increased and decreased to original rate. *C,* water gently stopped and started. Note respiratory inhibition without cardiac response.

When the sensory stimulation was strong or prolonged, both cardiac and respiratory inhibition occurred. Each effect, however,

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could be produced independently. If the stimulus was weak or brief, especially when applied to the skin, stomach, or spiral valve, only a cardiac response was elicited (Figs. *2A,* 3, *4B,* *5C* and *6B*). But a weak stimulus to the gill region frequently produced only respiratory inhibition. This occurred sometimes when the flow of perfusion water was altered (Fig. *7C*).

Mechanical stimulation of the sides of the pericardial chamber by stroking with a sharp needle gave marked cardiac inhibition (Fig. 4). Weak faradic stimulation of the posterior wall of the chamber for a few seconds frequently gave an inhibitory effect lasting a half minute or more. On the other hand, pinching the sides of the body at the level of the pectoral fins vigorously with the forceps for twenty-five seconds stopped the heart for a short period, but it escaped from complete inhibition while still being reflexly stimulated.

When the surface of the ventricle was pinched lightly with small forceps, it immediately contracted and then became completely inhibited in diastole for 30 seconds accompanied by inhibition of respiration in relaxation and by movements of the head. If the ventricle was allowed to beat against a sharp needle, it contracted quickly a few times almost stopping in systole, then became completely inhibited in diastole, generally associated with respiratory inhibition. If the needle was inserted carefully under the epicardium, there was no systolic inhibition or respiratory response, but complete diastolic inhibition and slow recovery. Reflex diastolic inhibition from mechanical stimulation of the ventricle was also obtained after the aorta had been cut. A weak brief faradic stimulus applied to the surface of the ventricle caused immediate fibrillation under the electrode and, when the stimulus was removed, marked diastolic inhibition. Reflex cardio-inhibition from the ventricle was also obtained with only the post-branchial branches of the fourth branchial divisions intact. With the vagi cut or the heart excised, mechanical or faradic stimulation of either the auricle or the ventricle did not inhibit in diastole, but faradic stimulation along the Cuverian duct was immediately effective in producing this type of inhibition.

Discussion

While the results reported above indicate that respiratory and cardiac inhibition usually occur together, they show no evidence that there is a dependence of the one on the other. Each may occur independently. Moderate or strong stimulation, however, at any point mentioned above, except the liver and the testis, invariably caused both cardiac and respiratory inhibition. The liver and testis apparently have no sensory supply, stimulation of which gives either cardiac or respiratory responses.

Although stimulation of the central end of the lateral line nerve cut either at the gill region or midway between the pectoral and pelvic fins, gave both inhibitory responses, neither MacWilliam (1885) nor Kolff (1908) obtained heart reflexes on stimulation of this nerve in teleosts. Parker (1909) noted a temporary cessation of respiration on applying pressure to the lateral line region of the elasmobranch *Mustelus canis.*

The cardio-inhibition obtained on mechanical or electrical stimulation of the surface of the ventricle was undoubtedly of reflex nature since it was often accompanied by respiratory inhibition and skeletal muscular activity, responses which in themselves indicate afferent fibers from the ventricle. None of these responses was obtained with all of the cardiac branches of the vagi cut. In a paper on the innervation of the heart of *Scyllium* (Lutz, 1930*a*), further evidence is presented which indicates that the afferent pathway is in the vagus.

That reflex cardiac and respiratory inhibition should be so easily evoked by sensory stimulation of almost every part of *Scyllium* is interesting in view of the well-developed chromaphil system found in elasmobranchs and the inhibitory effect of adrenaline on the perfused heart of *Scyllium* described by Macdonald (1925). A similar effect of adrenalin chloride on a sinus-auricle preparation of various species of skate (*Raia*) and the dogfish, *Squalus acanthias,* has been described by Lutz (1930*b*). In view of the poorly developed sympathetic system (Müller and Liljestrand, 1918) and the lack of vaso-constrictor nerves (Schoenlein and Willem, 1894, and Schoenlein, 1895) found in elasmobranchs, the writer suggests that these inhibitory responses may be the expression of an emergency function which serves the fish in case of injury, especially to the gill region, and he has discussed the idea elsewhere (Lutz, 1930*b*).

I wish to thank Dr. Reinhard Dohrn and Dr. Enrico Sereni for the many courtesies extended at the Zoölogical Station, Naples, and Edna B. Lutz for technical assistance.

Summary

1. Mechanical and electrical stimulation of various parts of the surface of the body and certain viscera resulted in cardiac and respiratory inhibition when the spinal cord was pithed posterior to the second vertebra in *Scyllium canicula.*

2. Faradic stimulation of the central end of the cut vagus, either

the cardiac branches or the visceral ramus, and the central ends of the cut hypobranchial and lateral line nerves produced reflex cardiac and respiratory inhibition.

3. Stopping or starting the perfusion water through the gills or suddenly altering its rate of flow resulted in respiratory inhibition and sometimes cardiac inhibition.

4. Mechanical or electrical stimulation of the surface of the ventricle produced reflex cardio-inhibition and sometimes respiratory inhibition.

5. When stimulation was strong or prolonged, both cardiac and respiratory inhibition occurred, but weak stimulation of the gills or pharynx frequently produced only respiratory inhibition, whereas weak stimulation of the skin or viscera often produced only cardiac inhibition.

6. Both cardiac and respiratory inhibition were obtained for over twenty-four hours when the gills were perfused; for over three and one-half hours without perfusion; and for over one hour with the aorta transected at the heart. These phenomena, therefore, are true reflexes and are not due to alterations in blood pressure in the medulla.

7. The emergency function of the inhibitory responses is pointed out.

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RESPIRATORY RHYTHM IN THE ELASMOBRANCH, *SCYLLIUM CANICULA*

BRENTON R. LUTZ

(*From the Zoölogical Station, Naples and the Physiological Laboratory of Boston University School of Medicine*)

The ease with which respiratory reflexes may be elicited by stimulation of the surface of the pharynx and gills in fishes led early investigators to look for a reflex control of respiration in these vertebrates. Schoenlein (1895) perfused the gills of *Torpedo* and *Scyllium* through the spiracle and found the respiratory rate to increase with increased flow of water until, with too great a flow, the regular rhythm ceased and ejection reflexes occurred. If the flow of water was stopped, there were a few respiratory movements until all the water in the gills was pressed out, when complete passive inhibition of respiration occurred. Since this author observed no signs of dyspnea during perfusion with oxygen-free sea water for twenty minutes, he came to the conclusion that the control of respiration was mainly reflex. Bethe (1903) perfused the gills of *Scyllium canicula* and *S. catulus* both with oxygen-free sea water and with sea water saturated with carbon dioxide, and found that the rate and strength of respiration remained unaltered for about forty minutes, after which it began to be slower and weaker. He also paralyzed the peripheral receptors of the gills and pharynx with cocaine and obtained a cessation of respiration. He therefore concluded that the gas content of the blood, which in the higher vertebrates plays such an important part, is without influence in fishes, and that breathing is caused by peripheral stimulation.

Since these conclusions contradicted the results of Duncan and Hoppe-Seyler (1893), who determined the level to which the oxygen content of the water could fall before signs of disturbed respiration occurred in fishes, Baglioni (1907–08) investigated the reflex respiratory mechanism in both teleosts and elasmobranchs. He found that the respiratory inhibition which occurred on stopping the flow of water was not absolute, and he believed it to be a reflex due to air in the pharynx. He also found that fishes which were allowed to swim freely in evacuated sea water showed a marked increase in respiratory rate, extreme dyspnea, and periodicity. He concluded that it is a general property of the central nervous system in all vertebrates to react to oxygen lack by increased irritability.

The present paper is concerned with the respiratory responses which occur when the flow of blood through the respiratory center is stopped, and also with the effect of altering or stopping the flow of water through the gills.

METHOD

Vigorous specimens of the dogfish, *Scyllium canicula*, averaging 300 grams, taken during the months of March and April from the Bay of Naples, were used. The fish was taken out of water and the fore-brain was separated from the rest of the brain by a transverse cut just in front of the optic lobes and destroyed. The spinal cord was transected at various levels between the third and twelfth vertebræ and pithed posteriorly in order to keep the fish quiet. The animal was then placed ventral side up on a fish holder and the gills were perfused through the mouth. Respiratory movements soon became regular. The heart was exposed through the ventral wall of the pericardial chamber and a small gold hook was put through the superficial tissue at the tip of the ventricle. By means of a thread and a light writing lever its movements were recorded. A similar hook passed through the anterior border of the third or fourth gill slit was attached to a thread, passed horizontally to a pulley, and up to a light writing lever for recording respiration.

The perfusion water was taken from three large glass bottles placed 60 cm. above the fish holder. The rate of flow was controlled by means of a glass cock on each outflow tube near the bottle, and another near the mouth of the fish.

In certain experiments the aorta was transected at its junction with the conus arteriosus.

RESULTS

A sudden alteration in the rate of flow of water to the gills evoked a reflex respiratory response and generally cardiac inhibition. The immediate effect of suddenly stopping and starting the flow or of increasing and decreasing the rate was respiratory inhibition, usually followed by one or more rapid and vigorous contractions of the gill muscles during which water was ejected from the mouth as well as from the gill slits. This ejection reflex occurred both when additional water was suddenly turned on and when the water was suddenly stopped (Fig. 1, *A* and *B*). A similar response to abrupt alteration in water supply to the gills occurred after the aorta had been transected to prevent any possible blood pressure effects through changes in water pressure on the gill vessels.

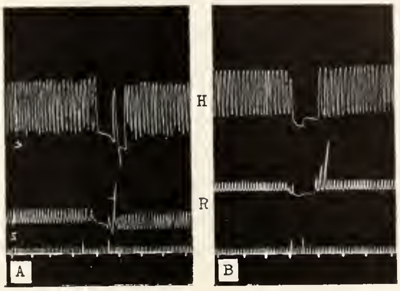

FIG. 1. Reflex cardiac and respiratory responses. In this and in subsequent figures the upper tracing is a record of the heart beat. The middle tracing records respiration. The large divisions on the time-signal record are ten seconds. *A*, perfusion of the gills suddenly stopped and started. *B*, flow of perfusion water quickly increased and decreased.

When the rate of flow was changed gently from 200 cc. per minute to 400 cc., there was an increase in both respiration rate and heart rate. If the flow was then reduced to the original, there was a return to the previous heart and respiratory rates (Fig. 2). In this case the heart rate before the change was 25 per minute, and the respiratory rate was 21. During a flow of 400 cc. per minute they were 42 and 27, respectively. A return to 200 cc. per minute gave 22 and 21, and a second increase to 400 cc. resulted in a heart rate of 42 and a respiratory rate of 31. Too great an increase, however, produced an irregularly inhibited rhythm of both the heart and the respiration.

When the flow of water was stopped for a period of several minutes, there was usually a temporary inhibition of both respiration and heart, followed by a slower rhythm which was sometimes very regular (Fig. 3) and sometimes showed a tendency toward periodicity (Fig. 4). With further continued lack of water the regularity of respiration was interrupted from time to time by generalized reflex muscular responses from the remaining parts of the central nervous system, during which both respiration and heart were irregularly inhibited. The medullary centers remained reflexly functional for over three and one-half hours.

The immediate result of transection of the aorta at the conus arteriosus was inhibition of both respiration and heart. But in a minute or two both began rhythmical activity although somewhat slower. Even without perfusion of the gills, regular respiratory movements occurred for a variable length of time. Figure 5*A* shows the

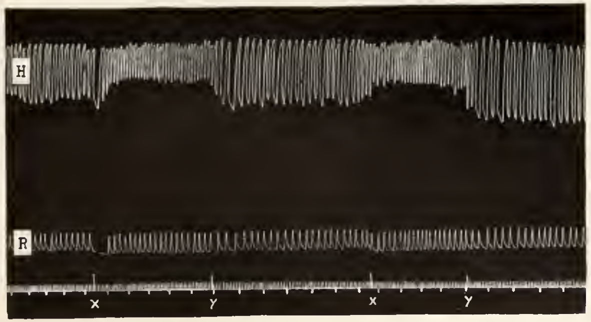

Fig. 2. Cardiac and respiratory responses to changes in the rate of flow of perfusion water to the gills. *X*, from 200 cc. per minute to 400 cc. *Y*, from 400 cc. to 200 cc.

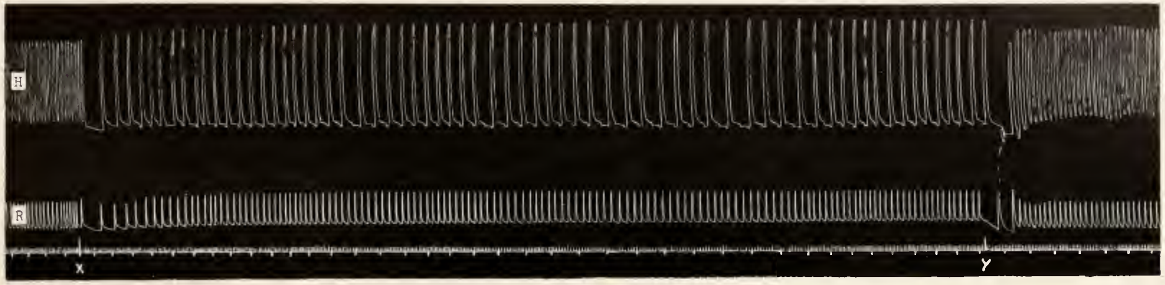

Fig. 3. Perfusion water to the gills stopped from *X* until *Y*, a period of 6 minutes and 32 seconds. Regular respiratory rhythm.

regular respiratory rhythm becoming periodic about ten minutes after transection of the aorta. Figure 5*B* was taken from the same fish fifty-three minutes after the aorta was cut. A periodic series of dyspneic respiratory movements occurred followed by a weaker and

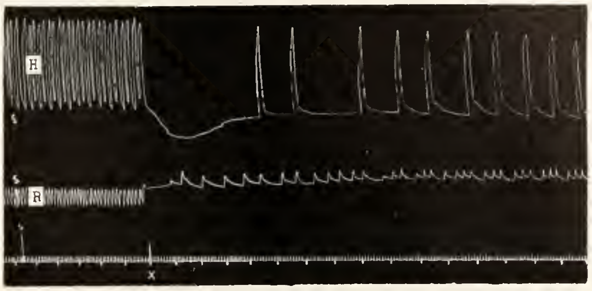

FIG. 4. Perfusion water to the gills stopped at *X*. Onset of periodic respiration after 90 seconds.

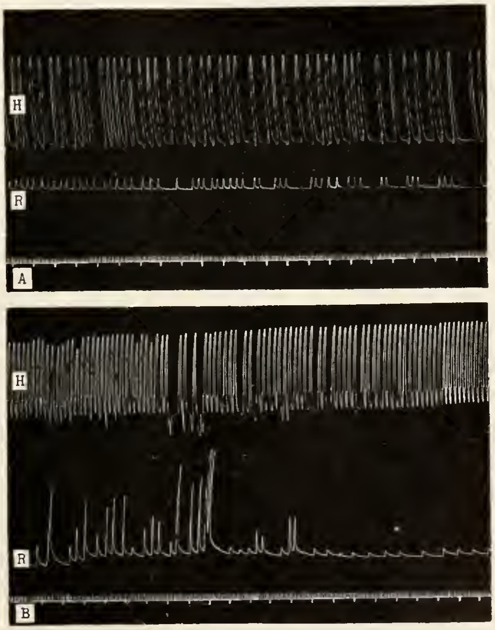

FIG. 5. Aorta transected at the conus arteriosus and perfusion of the gills stopped at the same time in a fish with the cord destroyed posteriorly from the fourth vertebra. *A*, record of heart and respiration beginning ten minutes after transection of the aorta, showing the onset of periodic respiration. *B*, records from the same fish beginning fifty-three minutes after transection, showing dyspneic periodicity giving way to weak, slow respiration.

more regular rhythm. The heart rate showed some irregular slowing but otherwise was generally more rapid, probably indicating a partial loss of vagus tone. In this fish the medullary centers remained reflexly functional for sixty minutes without a blood supply.

Discussion

The results reported above confirm Schoenlein (1895) insofar as an increase in the rate of flow of water to the gills caused an increase in respiratory rate. Springer (1928), working on *Mustelus canis* and *Squalus acanthias*, also reported that "the stronger the current the greater the rate of respiration." The marked, though rough, synchronism between the respiratory rate and the rate of the heart on altering the flow of water is difficult to understand, especially since the heart rate was changed about twice as much as the respiratory rate. It suggests a close nervous association between the respective medullary centers, although Lutz (1929) has found that each center may act independently. Inasmuch as accelerator nerves to the heart have not been demonstrated, the increase in heart rate accompanying increased respiration is probably due to inhibition of vagal tone. The vagus is known, under experimental conditions at least, to exert a continued influence on the heart (Lutz, 1930). Since *Scyllium* takes in water through the mouth on inspiration (Baglioni, 1907–08), a mechanism for increased respiration and heart rate during rapid swimming is clear.

That peripheral stimulation by water, however, is not necessary for continued regular respiration is obvious from Fig. 3. The inhibition on stopping the flow lasted only twelve seconds in this case, and ten seconds in the case illustrated in Fig. 4. This inhibition appears to be a transient reflex due to stimulation by collapse of the pharynx on stopping the flow of water. It occurred immediately, rather than after the water had been pressed out by several gill movements and air had entered, as suggested by Schoenlein (1895). Moreover, after the water had been expressed, a regular rhythm, rather than inhibition, was set up. It is apparent that, just as in the higher vertebrates, the rate of respiration is easily subject to peripheral control, but that a slow regular rhythm will continue without the usual peripheral stimulus.

The continuance of regular respiratory movements after transection of the aorta indicates an automaticity of the respiratory center without a blood supply. A rhythmic discharge from the cord in higher vertebrates has been found to occur under asphyxial conditions by Langendorff (1887) and by Brown-Séquard (1893), but the resulting movements are not considered to be true coördinated respiratory processes. In several teleosts Kolff (1908) noted the continuance of breathing movements for fifteen minutes after the heart had been removed. In the case of *Scyllium* with a transected aorta the regular rhythm which occurs at first, the onset of periodicity, and the subsequent dyspnea parallel the results of Baglioni (1907–08) with intact fishes in evacuated sea water. The usual explanation of periodicity involves alternate changes in the gas content of the blood flowing through the respiratory center. However, it appears that a periodic rhythmic discharge may occur without a blood supply, and therefore the cause for the periodicity must lie primarily within the nerve cells of the center.

Since it is impossible in the fish to cut the afferent fibers from the gills without destroying the motor pathways for respiration, conclusive proof of the automaticity of the respiratory center will be difficult to obtain. However, since rhythmic respiration continued after the water was stopped and the blood supply to the medulla interrupted, one may infer that, in fishes as in mammals, the respiratory center is autonomous. Furthermore, as in mammals, it is influenced by the gas content of the blood and by the peripheral stimulation.

SUMMARY

1. The respiratory and cardiac responses to stopping and starting the water supply to the gills, to increasing and decreasing the rate of flow, and to complete interruption of the blood supply to the medulla of *Scyllium canicula* were recorded.
2. Sudden stopping and starting or increasing and decreasing the water supply evoked brief respiratory inhibition and sometimes ejection reflexes.
3. An increase in the rate of flow of sea water to the gills caused an increase in respiration and heart rates. A decrease caused the reverse. Too great an increase, however, evoked irregular inhibition.
4. Stopping the flow of water to the gills evoked brief inhibition of respiration followed by a regular rhythm at first, which sometimes became periodic and finally was interrupted by generalized muscular activity. The medullary centers remained reflexly functional for over three and one-half hours.
5. Interruption of the blood supply to the medulla when there was no water supply to the gills was followed by regular respiration at first, then periodicity and dyspnea. The medullary centers remained reflexly functional for over sixty minutes without a blood supply.

6. It is concluded that the respiratory center is autonomous, but that it is influenced by the gas content of the blood and by peripheral stimulation.

I wish to thank Dr. Reinhard Dohrn and Dr. Enrico Sereni for the many courtesies extended at the Zoölogical Station, Naples, and Edna B. Lutz for technical assistance.

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THE MEASUREMENT OF THE RESPIRATORY EXCHANGE OF AQUATIC ANIMALS

ANCEL B. KEYS

Scripps Institution of Oceanography, La Jolla, California

Introduction

Recognition of the importance of studying the gas exchange of aquatic animals is attested to by the large number of papers which have appeared on this subject. The present article is the result of a series of investigations on the accuracy of various methods previously described for the measurement of this exchange and presents a method which has proven satisfactory for a rather wide variety of purposes.

Methods of Measuring the Gas Exchange of Aquatic Animals

There are three general types of methods of measuring the respiratory exchange of aquatic animals. The simplest method consists merely in determining the time rate of change in the gas contents of a closed vessel of water containing the animal under investigation. Details and applications of the method are given by Humboldt and Provençal (1809), Henze (1910*a*), Montuori (1913), Krogh (1916), McClendon (1917), Powers (1922), and Bruce (1926), among many others. The fact that oxygen is diminishing and carbon dioxide and nitrogenous excretions are accumulating in the water during the period of the determination renders this method unsatisfactory. Further objections will appear in the fourth section (" Method finally adopted . . .") of the present paper.

Applications of the Regnault (Regnault et Reiset, 1849) principle to aquatic respiration constitute the second general method. It has been used in various forms by Jolyet et Regnard (1877), Gréhant (1886), Zuntz (1901), Bounhiol (1905), and Gardner and Leetham (1914). The many complications and sources of error in this method have led to criticisms of it by Krogh (1916, p. 50) and Henze (1910*b*, 1927).

The third method for measuring the gas exchange of aquatic organisms involves a flowing water system. Briefly, a measured amount of water of known gas content is passed, in a given period of time, through a vessel containing the animal, and samples of the water are collected and analysed after passing through this respiratory chamber. The application of this method has been almost entirely limited to special cases where a sort of artificial respiration is needed, such as in the case of narcotized fish, etc. Thus Winterstein (1908) used it to measure the strictly branchial respiration, while Ege and Krogh (1914) and Gaarder (1918) used it to determine the oxygen uptake of fishes under urethane narcosis. Hall's (1929) use of this method is open to criticism, as I have pointed out elsewhere (Keys, 1930*a*).

The Investigation of Methods in this Laboratory

Of the three general methods mentioned above, only the first and third were considered. The first method was subjected to a thorough trial in four different arrangements of apparatus. The test animals used in each case were marine fishes (*Fundulus parvipinnis*). A number of difficulties which seemed to be inherent in the method were found. It is not necessary to describe these experiments in detail; one particular source of difficulty was stratification in the gas content of the water in the respiratory vessel.[1]

Having taken into account as many of the sources of error as possible, the final test was made of attempting to duplicate results for the oxygen consumption of individual fishes by this method. In but few of these experiments was the oxygen consumption, as indicated by one determination, duplicated within 15 per cent by a second.

At this juncture experiments with the third method were begun. The apparatus arrangement which had been used by Ege and Krogh was used first. Various modifications of apparatus and technique were subsequently tried until the final method, which has been adopted as standard in this laboratory, was evolved.

It was found that the abstraction of the sample of the incoming water in the manner in which it was done by Ege and Krogh (see Krogh, 1916, p. 51) and, so far as I know, by all others who have used the flowing water method, not infrequently leads to serious errors due to stratification of the water through the respiratory chamber. The most serious drawback to the method of Ege and Krogh was the use of narcotics. Fortunately, however, it was found that the normal, unanesthetized animal could be used without difficulty. A large number of fishes, representing six different species, were observed in small respiratory chambers similar to those used by Gaarder and in every case the fish became perfectly quiet in a few minutes and remained so for many hours so long as a sufficient flow of water was provided.

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[1] In some of these earlier experiments I had the assistance of Mr. Rolland Main, whose help is gratefully acknowledged.

Method Finally Adopted for Fishes and Other Macroscopic Aquatic Animals

Figure 1 shows the apparatus used for experiments on the gas exchange of fishes. The respiratory chambers *A* and *B* each contain a single fish and are chosen to be of such size that, although almost the whole of the water passing through the chamber comes into contact with the animal and is available to it for respiration, the fish is not ordinarily in contact with the walls of the chamber and is free to move about to some extent. If the fish is not disturbed after it is placed in the chamber, and if the current of water is properly adjusted at a constant flow, the fish quickly assumes a position facing the incoming stream and thereafter almost perfect quiescence is the rule, the only visible mus-

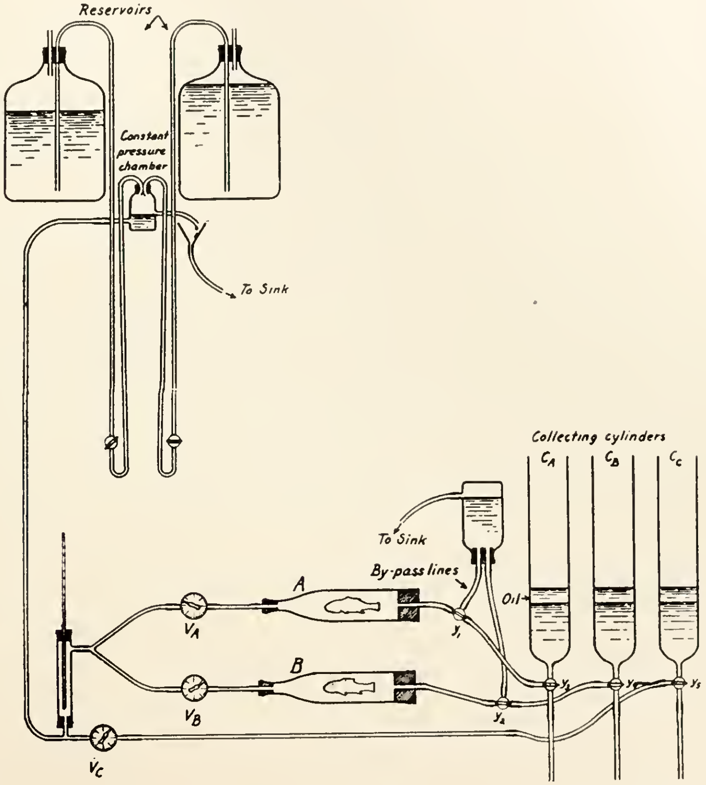
Reservoirs
Constant pressure chamber
To Sink
Collecting cylinders
C_A
C_B
C_C
To Sink
By-pass lines
A
B
Oil
V_A
V_B
V_C
Y_1
Y_2
Y_3
Y_4
Y_5

Fig. 1. Apparatus for determining metabolic rates of aquatic animals. The proportionate sizes shown are for the arrangement of the apparatus for studies of the respiratory exchange of small fishes such as *Fundulus*.

A, B, respiratory chambers; Y_{1-5}, 3-way stopcocks; $V_{A,\ B,\ C}$, needle valves.

cular activity being the respiratory movements of the operculum and the leisurely motion of the pectoral fins which serves to keep the fish oriented in the current.

The collecting cylinders C_A and C_B receive the whole of the water which passes through respiratory chambers A and B respectively except when the water is by-passed through the by-pass lines. The by-pass chamber is so designed that it gives approximately the same back-pressure as the collecting cylinders when they are in use and, accordingly, with a given setting of the needle valves V_A, V_B, and V_C, the rate of flow through the system is the same, irrespective of whether the water is being collected for analysis or by-passed. The constant pressure chamber insures the head pressure remaining the same, no matter what the amount of water in the reservoirs. If the collecting cylinders are immovably fixed in position, the back pressure will increase as the cylinders fill, with a resulting decrease in the rate of flow; this can be taken care of by suspending them by long springs or by manually adjusting their level from time to time during each collection.

Collecting cylinder C_C receives the control water for the determination of the gas content of the water before it is respired by the fishes. The possibility of error due to stratification in the reservoirs is thus eliminated by a constant fraction of all the incoming water being diverted to the control. If stratification persists in the collecting cylinders it is easily broken up by gentle shaking or stirring of these last; this, of course, does not disturb the animals in the respiratory chambers.

It will be noted that no provision is shown for maintaining constant temperature. To secure the best results the whole apparatus should be mounted in a constant temperature room. Failing this, the tubing connecting the needle valves with the respiratory chambers should be lengthened into long coils and these last, with the attached respiratory chambers, should be suspended in a constant temperature water bath. In this case the thermometer chamber should be placed inside the bath at the end of one of the coils.

All water for gas analysis should be collected under at least three centimeters of the best quality saturated-hydrocarbon mineral oil. The gas content of the water is readily determined by standard methods, although the determination of carbon dioxide in sea water presents some difficulties. For oxygen the best method is undoubtedly the Winkler (1888) iodometric volumetric method. This method is given in detail in the American Public Health Association manual (3rd edition, 1917), by Treadwell and Hall (1928), and by Sutton (11th edition, 1924). The determination of dissolved oxygen in sea water is fully described by Jacobsen (1921). The determination of carbon dioxide in the water may be made by the Van Slyke manometric method which will be found in the articles by Van Slyke and Stadie (1921), Van Slyke (1927), and Van Slyke and Sendroy (1927). An alternative method for determining CO_2 in sea water and brackish waters which utilizes pH determinations is given by Bruce (1924).

It should be pointed out that simple modifications, such as in the size of various parts of the apparatus, enable the flowing water method, as presented in this paper, to be used for very small animals. A correspondingly small volume of water must be used, and in this case the Van Slyke apparatus may be used for both oxygen and CO_2 in water volumes as small as one or two milliliters. The micro-Winkler method for oxygen (Thompson and Miller, 1928, and Snoke, 1929) may be used with samples as small as five milliliters. In all determinations, of course, duplicate analyses should be made.

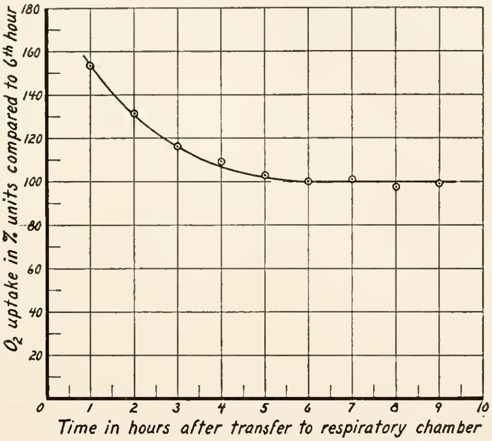

FIG. 2. Serial determinations of the oxygen uptake of *Fundulus parvipinnis,* showing initially high values and the basal or standard rate of exchange attained after several hours. Average curve for ten individuals.

One of the most important considerations in determinations of the gas exchange of the fishes is the time element. In all experiments reported in the literature the determination was begun as soon as the animal had ceased violent muscular activity, although in some cases even this precaution was omitted. Usually a single determination was made, the period of this determination varying in different cases from ten minutes to three or four hours; in a very few cases a longer period was used. With this in mind I wish to call attention to Fig. 2. The points in this graph were obtained from serial determinations on ten different specimens of *Fundulus parvipinnis*. In order to bring out the general trend in the oxygen consumption of these fishes from hour to hour after transfer to the respiratory chamber, I have reduced all the data to percentage values, taking the oxygen consumption at six hours as the point of 100 per cent. It is clear that a constant rate of oxygen consumption is attained about five or six hours after transfer of the fishes to the respiratory chambers, and that determinations made before this time had elapsed would have overestimated the standard metabolism.

The appearance of the graph for *Fundulus parvipinnis* is characteristic of all the species of fishes which have been studied in this laboratory.[2]

Figure 3 shows an unusually complete series of determinations on a single specimen of *Girella nigricans* and depicts the constancy of results which may be obtained by the use of the method described in this paper.[3] I wish to make it clear that in all cases the fishes were very quiet after the first quarter or half hour in the respiratory chambers. In spite of this fact, the gas exchange did not reach a constant level until from four to six hours. In several hundred of these serial determinations this was found to be invariably the case. The results of a series of determinations on a specimen of *Clinocottus analis* are shown in Fig. 4.

These same general results were obtained in large as well as in small respiratory chambers and were found when the fishes were in total darkness as well as in ordinary daylight. They point to the conclusion that, in order to be sure that the determinations represent "standard" or anything like "basal" metabolism, it is necessary to wait five or six hours after the fish has been placed in the apparatus before beginning the final determinations. These findings throw doubt on the validity of any method in which the period of the determination of the gas exchange begins shortly after the transfer (and handling incident to this) of the animal to the vessel which is used for the experiment.

The procedure recommended in beginning gas exchange experiments on an animal form which has not previously been investigated, is to make the determinations serially, which is very simple with the apparatus described in this paper, until constant gas exchange is reached. Experiments in this laboratory indicate that, in the case of

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[2] These species are *Girella nigricans, Fundulus parvipinnis, Clinocottus analis, Leptocottus armatus.*

[3] I was fortunate in having the collaboration of Mr. N. A. Wells in the application of the method to *Girella* and to *Clinocottus analis.*

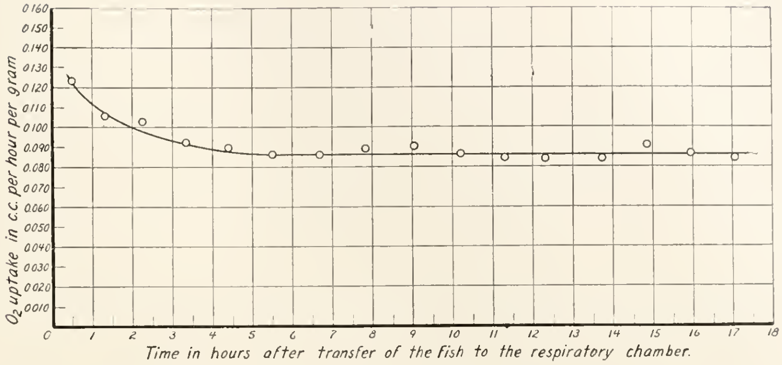

FIG. 3. Serial determinations of O_2 uptake of *Girella nigricans,* fish no. G_4, weight 40 grams. Three days starvation. Temperature 16.7° C.

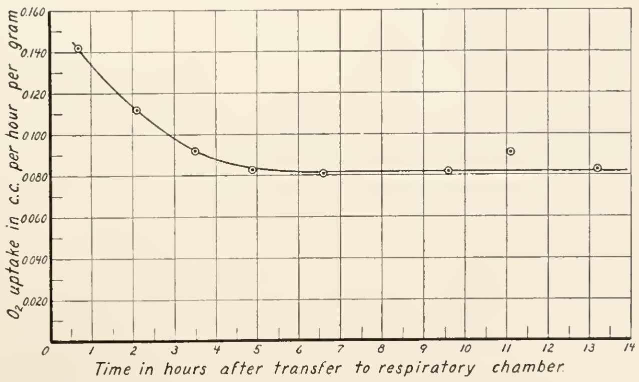

FIG. 4. Serial determinations of O_2 uptake of *Clinocottus analis,* fish no. C_5, weight 37 grams. Temperature 20.0° C.

most fishes, it is perfectly safe simply to transfer the animals to the apparatus, allow the system to run (through the by-pass lines) for six hours, and then make two or more determinations covering, let us say, about two hours as a minimum. If these determinations check reasonably well (as is ordinarily the case), it may be assumed that the results (or the mean of the results) represent standard metabolism.

The characteristic appearance of the time graph of the gas exchange demands some comment. The elevated gas exchange which persists for a matter of some hours is not due to muscular activity, at least no such activity is visible. It seems certain that at least part of it is due to the payment of an oxygen debt contracted by the fish in its struggle incident to being placed in the apparatus.[4] Another possible explanation for the phenomenon might be found in the fact that, whereas a certain minimum stimulus is necessary to bring about a nervous impulse and consequent muscular action (" all or none " principle), sub-minimal stimuli will cause a marked increase in the gas exchange of nervous tissue at least (Winterstein and Hirschberg, 1927).

I have already indicated how, by making serial observations, it is a simple matter to demonstrate that the method developed here is capable of yielding very consistent results. In a number of cases determinations of the oxygen uptake of certain fishes were repeated after a lapse of from two or eight days. The results of these experiments are summarized in Table I.

The average discrepancy between first and second determinations is 2.8 per cent. In each case the oxygen uptakes in the table represent the average of two or more determinations. I should warn the reader that such good results are not to be expected until the investigator has had considerable experience with the technique. Good results require unremitting vigilance in the maintenance of constant physical and physiological conditions.

It will be noted that in Fig. 1 there are two respiratory chambers shown. This is a highly desirable feature as it enables the investigator to compare two animals, even though it is not possible to maintain standard conditions. Systematic errors in the determinations are frequently detected if two animals are used in this way. Of course, if the experimenter desires, three, or even more, respiratory chambers with their respective collecting cylinders, valves, etc., may be used, but the services of a number of assistants will be found to be necessary if this is done.

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[4] The period of payment of oxygen debt is usually not nearly so prolonged in mammals (Hill and Lupton, 1922; Campbell, Douglas and Hobson, 1920; Krogh and Lindhard, 1920) as is indicated in the experiments on fishes but, as Barcroft (1925) points out, the duration of the debt is directly related to the adequacy of the blood supply, and we know (Krogh, 1929) that the capillary blood supply to the muscles of fishes is meagre compared to that of the warm-blooded animals. We should expect the payment of the oxygen debt of any cold-blooded animal to be slower than in a warm-blooded animal in the same way that all the vital processes proceed at a relatively slow rate in the cold-blooded animals.

TABLE I

Species	Fish No.	Weight	Date	Temperature	O_2 uptake per hour
		grams		°C.	*grams*
Fundulus	M_{35}	4.3	12/26/28	20.0	0.207
"	"	"	12/31/28	"	0.214
"	M_{43}	9.1	6/25/29	"	0.161
"	"	"	7/ 2/29	"	0.159
"	M_{44}	4.5	6/25/29	"	0.209
"	"	"	7/ 2/29	"	0.202
"	M_{50}	7.3	6/28/29	"	0.194
"	"	"	6/30/29	"	0.208
"	M_{51}	4.4	6/28/29	"	0.280
"	"	"	6/20/29	"	0.281
Girella	A_7	257	12/ 5/29	20.0	0.0615
"	"	"	12/10/29	"	0.0623
"	A_9	210	12/12/29	18.0	0.0586
"	"	"	12/20/29	"	0.0607

There are a number of precautions which should be taken. The water used should always be filtered and well aërated before use, but should never be supersaturated. The whole system should frequently be tested for leaks and should never be used with air bubbles trapped in any part of the apparatus. The animals used should always be starved long enough before the determination to insure that the digestive tract is empty at the time of the experiment.

Applications of the method described here to particular problems involving a study of the gas exchange of aquatic animals are given in a paper by the author (Keys, 1930*b*) now in press, as well as in other papers in the course of preparation at the Scripps Institution.

CALCULATION OF RESULTS IN RESPIRATION EXPERIMENTS

The calculations for the determination of oxygen and carbon dioxide by the Van Slyke manometric apparatus are given in the papers of Van Slyke already cited. The calculations for oxygen by the Winkler method are given in the references on this method listed above.

Tables of the normal saturation values of water with respect to oxygen are given by Fox (1907), Jacobsen (1921), and Harvey (1928). Fox's data (given also by Harvey) were obtained by equilibrating water of various salinities with atmospheric air at different temperatures. Jacobsen's data are for air minus water vapor and carbon dioxide. Salinity may be converted into chlorinity by the use of Knudsen's formula

$$\mathrm{Cl} = \frac{\text{salinity} - 0.030}{1.8050}$$

where *Cl* is the chlorine equivalent of the chlorine, bromine and iodine in the sea water, and salinity is the total weight of salts (carbonates as oxides and organic matter ignited) in grams per 1000 grams of sea water.

Summary

The methods previously used for the measurements of the gas exchange of aquatic animals are discussed. Of the three general methods, two were subjected to careful investigation in experiments on marine fishes.

The method finally developed for the study of aquatic respiration is described in detail and typical results obtained by its use are given. It is shown that the method yields consistent results which may be accepted as measures of standard metabolism.

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RATE OF HISTOLYSIS OF ANURAN TAIL SKIN AND MUSCLE DURING METAMORPHOSIS

H. J. CLAUSEN

Department of Zoölogy, State University of Iowa

The factors responsible for the atrophy of the larval anuran's tail will no doubt require considerable further work before a complete and adequate explanation can be derived. The main results and conclusions reached to date may be briefly reviewed as follows: Barfurth (1887) first suggested that the growing urostyle occluded the adjacent dorsal aorta and so cut off the blood supply to the tail. Mercier (1906) later found phagocytes in the muscle masses of the tail which contained carmine granules previously injected into the dorsal lymph sac. This indicated that the occlusion of the aorta due to urostyle growth must be only a partial one. Bataillon (1891) studied the blood vessels of normal and atrophying tails and described a change in vascular distribution (notably of the capillaries) in the latter. More recently, Morse (1918) and Bradley (1922) have reiterated Barfurth's explanation and suggest that the reduced blood supply to the tail enables the CO_2 and various acid metabolites to accumulate. The result is the activation of the autolytic enzymes with consequent histolysis and general atrophy of the tail. Morse also suggested that a possible lowering of the blood pH might be the fundamental causative factor involved.

A new conception regarding the cause of anuran tail atrophy was evolved through the work of Helff (1928), which demonstrated that larvæ in which the anlagen of the urostyles had been extirpated exhibited typical tail atrophy during subsequent metamorphosis. The growth of the urostyle and the partial occlusion of the dorsal aorta could not, therefore, be looked upon as the fundamental factors inducing tail atrophy. Simultaneously, with this finding, Lindeman (1929) showed that tail skin transplanted autoplastically to the backs of larvæ underwent rapid and characteristic histolysis at the same stage of metamorphosis at which the integument of the tail normally degenerates. Back-skin grafts, previously transplanted to the tail, failed to undergo degeneration during larval involution. Helff and Clausen (1929) were likewise able to demonstrate practically the same results using tail-and back-muscle transplantations. It was found in this connection that although back muscle transplanted to the tail underwent 54 per cent reduction in volume due to atrophy during metamorphosis, similar sized grafts of tail muscle previously transplanted to the back were completely obliterated during the same time interval. The above results on skin and muscle transplantations suggested the probability that the various tissues of the tail are especially susceptible to histolytic reactions and that such reactions are the result, perhaps, of the presence of specific histolytic substances in the blood or of a general lowering of the pH of the latter during larval transformation.

During the course of Lindeman's (1929) work, the writer's attention was called to the fact that skin grafts placed near the anterior-posterior center of the larval tail were found to undergo a gradual migration towards the base of the tail. It was observed, however, that while the distance between the graft and tip of tail remained practically unchanged, the space between the base of the tail and the graft was constantly reduced as tail atrophy proceeded. Schubert (1926), during the course of work involving the transplantation of hind-limb buds to the tail, noted that the developing hind-limb transplants appeared to move progressively anterior as histolysis of the tail occurred during larval involution. The results of Lindeman and of Schubert both suggested the possibility that the anterior regions of the larval tail undergo histolysis at a considerably greater rate than is true of more posterior regions. The present paper records the results of experiments which were devised to test out the veracity of this assumption. In general the experiments consisted of the autoplastic, reciprocal transplantation of muscle and skin grafts of the back and tail, the transplants of tissue from the latter structure being taken from various longitudinal levels.

The experimental part of the present work was completed at the Iowa Lakeside Laboratory, Milford, Iowa, during the summer of 1929. The writer is greatly indebted to Dr. O. M. Helff for suggesting the problem and for his valuable suggestions and criticisms during the course of the work.

MATERIAL AND METHODS

The stock used for all operations were large *Rana pipiens*' larvæ, obtained from small ponds in the vicinity of Spirit Lake, Iowa, during the months of June and July, 1929. The animals when taken to the laboratory, were placed in large battery jars and fed fresh *Spirogyra* each day. The individuals selected from this stock ranged from 60 to 100 mm. in body length, with hind limbs between 4 and 15 mm. in length. These animals were normal larvæ and remained unchanged in the laboratory as typical larvæ for at least one week following the operations.

All animals used for transplantation purposes were anæsthetized in a 0.05 per cent aqueous solution of chloretone. The time required for anæsthetization by this method varied from five to ten minutes, depending somewhat on the size of the animals used and the temperature of the solution itself. The anæsthetized animals usually remained inactive for a period varying from one to two hours, thus giving sufficient time to allow the muscle grafts and the cut edges of the integumentary transplants to adhere properly.

The technique used in making the muscle transplantations was briefly as follows: A flap of the integument was first laid back and a small piece of muscle removed and placed in a few drops of lymph in a watch crystal. This piece was then accurately shaped under a dissecting microscope so that a cube of tissue, eight to twelve cu. mm. in volume, was prepared for transplantation. In transplanting, a flap of integument was likewise laid back from the region to receive the transplant, the underlying musculature lightly scraped, and the cubical muscle transplant then placed on this surface. The flaps of integument were now replaced and the animal placed in shallow water, with the operated areas exposed to the air so as to hasten the adherence of the cut edges of the skin flaps. All operations were autoplastic and reciprocal, *i.e.*, from tail to back and back to tail of the same individual.

The usual method of skin transplantation was also employed. Following the anæsthetization, integument from four regions of the tail was removed and each portion of integument placed in a watch crystal. The four pieces of integument were now shaped in the form of rectangles so that they corresponded in length and width one with another. The integument from four adjoining medial regions of the back was next removed and similarly shaped. The tail-skin grafts were finally transplanted to the wound areas on the back, and the back-skin grafts transplanted to the denuded areas of the tail. Following the usual exposure of the grafted areas to the air, the individuals were submerged in water to recover from the effects of the anæsthetization. Upon recovery, the operated individuals were placed in individual battery jars and maintained under constant laboratory conditions. Daily observations were made on all operated animals in regard to the process of healing, histolytic condition of the transplants, changes in form and size of the grafts, and the onset and progress of metamorphosis.

RESULTS

1. *Transplantation of Tail and Back Muscle*

The purpose in making autoplastic transplantations of muscle secured from the anterior, posterior, and two intermediate regions of the tail to the backs of larvæ was, primarily, to see whether or not muscle tissue from one region of the tail would undergo normal atrophy before that of other tail regions. The tail-muscle tissue to be transplanted was obtained from the side of the tail at either a posterior, anterior, or from one of two intermediate areas (text figure 1, areas *A*, *B*, *C*, *D*).

The muscle, after being carefully shaped and measured as previously described, was then transplanted, autoplastically, to a position slightly to one side of the mid-dorsal line on the back. (Text figure 1, *E*.) This position was selected to insure adequate contact of the graft with the underlying back muscle, since musculature of the mid-dorsal region of the back is very thin and rather poorly vascularized. The reciprocal transplantation of back muscle to the tail of the same individual was made in a similar manner for the purpose of showing possible differences in histolytic influence of the two foreign host regions on their respective transplants. The back muscle transplants, however, were always obtained from one region of the back and transplanted to the middle of the tail.

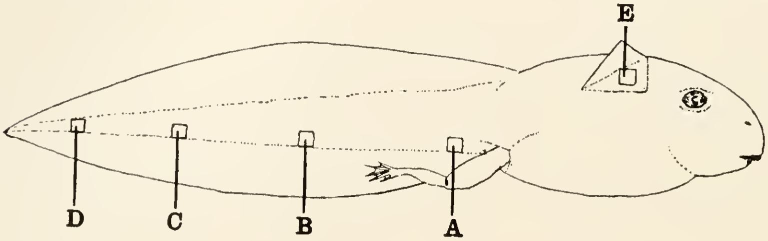


FIG. 1

One hundred and twenty-one successful reciprocal transplantations of tail and back muscle were obtained. The percentage of abnormal grafting proved to be less than one per cent. Table I represents the essential volume reduction data, due to histolysis during metamorphosis, for the four series of muscle transplantations. Series *A* represents those individuals in which the tail-muscle transplant was removed from the most anterior region of the tail; series *B* and *C* representing cases in which the muscle transplants were obtained from the intermediate tail regions; while series *D* were individuals whose muscle transplants were taken from a posterior region of the tail. The series therefore correspond in lettering to the operative areas *A*, *B*, *C*, and *D* as indicated in text figure 1. The various percentage reductions are based on the average volume reductions recorded at eleven-, sixteen-, and twenty-two day intervals following the operative procedures. The histological appearance of the grafts, sixteen days following transplantation, seems to compare favorably with that as described by Helff and Clausen (1929) for grafts examined fifteen days following transplantation.

TABLE I

Histolysis of Tail- and Back-muscle Transplants during Metamorphosis

Series	No. of Cases	Type of Transplantation	Average Volume Reduction		
			11 days	16 days	22 days
			per cent	*per cent*	*per cent*
A..........	31	Tail to back	47	79	98
		Back to tail	20	30	30
B..........	27	Tail to back	44	77	94
		Back to tail	20	35	35
C..........	31	Tail to back	40	70	92
		Back to tail	20	38	40
D..........	32	Tail to back	32	65	88
		Back to tail	20	38	38

An analysis of the data as represented in Table I will show that the tail-muscle transplants are markedly more susceptible to histolysis than is true of the back-muscle transplants. This is of even more interest when we consider that the latter transplants were surrounded by the atrophying musculature of the tail, while the tail-muscle transplants were in contact with back musculature which undergoes a lesser degree of resorption. Of primary interest, however, are the results of the muscle transplants obtained from different regions of the tail. Examination of the data will show that at all time intervals examined, the more anterior muscle grafts underwent a greater average percentage of atrophy than was true of more posterior muscle grafts. This would indicate that the anterior musculature of the tail is more susceptible to histolytic influences than is true of more posteriorly located muscle.

2. *Reciprocal Tail and Back Integumentary Transplantations*

The purpose of transplanting integument from various regions of the tail to the back was mainly to determine whether or not more anterior tail integument would exhibit a more rapid histolysis as compared with integument obtained further posterior on the tail. The reciprocal transplantation of back integument to the tail was primarily to see whether or not such integument would fail to undergo resorption during larval involution, as previously pictured and stated by Lindeman (1929).

The integumentary tail grafts were obtained from four regions of the tail (1, 2, 3, 4, text figure 2). These grafts will henceforth be spoken of as tail-skin transplants number 1, 2, 3, and 4, respectively. The integument, after being removed, was carefully shaped to form 2 × 7 mm. rectangles in order that the area reduction of this standard-sized graft could be accurately determined following subsequent transplantation and larval involution. Pieces of integument were next removed from four separate regions on the back and likewise shaped to conform to a 2 × 7 mm. rectangle. The tail-skin transplants were now grafted to the wound areas left by the removal of the back-skin transplants (text figure 3). Four types of orientation regarding the linear sequence of transplanting the tail-skin were made. In some cases tail-skin transplant number 1 was grafted to the most anterior

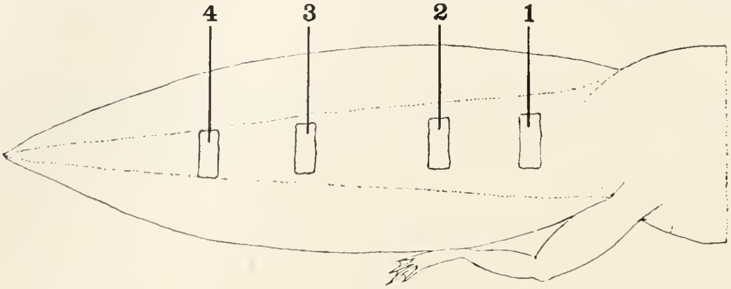


FIG. 2

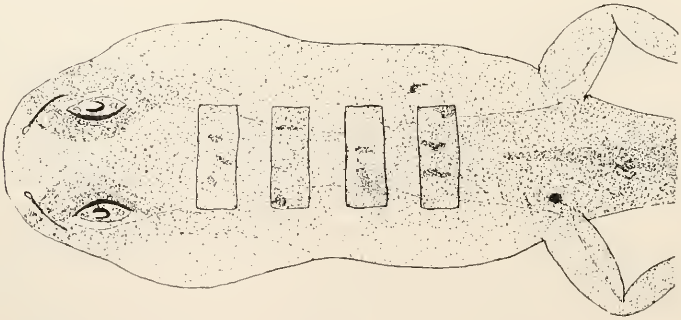


FIG. 3.

of the wound areas of the back, while tail-skin transplants number 2, 3, and 4 were grafted to respective posterior wound areas. This arrangement is spoken of as the 1-2-3-4 sequence. Where the reverse arrangement was made during transplantation (tail-skin transplant number 1 to the most posterior wound area, tail-skin transplant number 2 to the adjacent anterior wound area, etc.) the arrangement is spoken of as the 4-3-2-1 sequence. In addition to the above two arrangements of the four transplants, the sequences of 3-4-1-2, and 2-1-4-3 were also employed. The four back-skin transplants were likewise grafted to the wound areas on the tail using the same four sequences in the linear arrangement of the grafts.

Subsequent observation involved the measuring of the linear dimensions of the eight integumentary grafts of each animal at eleven- and sixteen-day intervals following the transplantation date. In this manner a fairly accurate estimate of the histolytic rate of the various transplants could be determined as metamorphosis took place. The essential data for the various series are represented in Table II. The

TABLE II

Histolysis of Tail and Back Integumentary Transplants during Metamorphosis

Series	Type of Transplantation	Average Surface Area Reduction							
		8th Day Observation				16th Day Observation			
		Tail-skin Transplant Number *				Tail-skin Transplant Number *			
		1	2	3	4	1	2	3	4
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
A Y (1–2–3–4) 22 cases	Tail to back	40	30	0	0	60	45	20	0
	Back to tail	0	0	0	0	20	15	0	0
B Y (4–3–2–1) 24 cases	Tail to back	25	25	0	0	50	45	0	0
	Back to tail	0	0	0	0	20	10	0	0
C Y (3–4–1–2) 20 cases	Tail to back	25	10	0	0	60	50	25	0
	Back to tail	0	0	0	0	30	15	5	0
D Y (2–1–4–3) 22 cases	Tail to back	40	30	5	0	70	50	30	0
	Back to tail	0	0	0	0	35	20	0	0

* The numbers 1, 2, 3, and 4, as they refer to the *back to tail* transplantations, are used to indicate the respective wound areas of the tail to which the grafts were transplanted.

data consist of the results of observation on the eight transplants, each, of eighty-eight larvæ. The most significant finding as evidenced by these results is that tail-skin transplant number 1 always showed a comparatively greater histolytic rate, while tail-skin transplants number 2, 3, and 4 showed progressively lesser speeds of atrophy. This gradient in the histolytic rate was found to hold true regardless of the linear sequence in which the transplants had been placed on the back. In other words, the particular location of any one transplant on the back was of no consequence to the rate of histolysis occurring. This point may be more easily understood by reference to Figs. 1, 2, 3, and 4, Plate 1. Further measurements of the tail-skin transplants at a time when transplant number 1 had been entirely obliterated, showed that transplants number 2, 3, and 4 had been reduced on the average but 70, 50, and 20 per cent respectively, in surface area. The difference in histolytic rate of the four transplants at the sixteen-day observation period is quite evident from histological evidence as pictured in Fig. 5, Plate 1.

FIGS. 1, 2, 3, 4. Illustrating the appearance of the four tail-skin transplants autoplastically transplanted to the back. The sketches were made at a time when tail-skin transplant number 1 was considerably histolyzed. In each figure transplant number 4 is still unchanged in external appearance and size and represents the size of all four tail-skin transplants at the time of operation. 1, 2, 3, and 4, tail-skin transplants obtained from regions 1, 2, 3, and 4, respectively, of the tail; *BI*, integument of the belly.

FIG. 1. Transplantation sequence 1–2–3–4.

FIG. 2. Transplantation sequence 4–3–2–1.

FIG. 3. Transplantation sequence 3–4–1–2.

FIG. 4. Transplantation sequence 2–1–4–3.

FIG. 5. Histological section through back integument including the four tail-skin transplants at the stage illustrated in Fig. 1. 1, 2, 3, and 4, portions of section composed of the respective four tail-skin transplants showing degrees of histolysis corresponding to the macroscopic reduction in size; *E*, epidermis; *SS*, stratum spongiosum; *SC*, stratum compactum; *CT*, subcutaneous connective tissue.

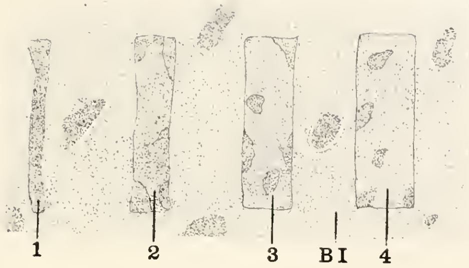
FIG. 6. Section through tail-skin graft on the belly and adjacent belly-skin as illustrated in FIG. 8, *TM*. The tail-muscle transplant is omitted in the figure. *BS*, belly-skin; *TG*, tail-skin graft; *x*, point of connection between belly-skin and tail-skin transplant.

FIG. 7. Section through tail-skin graft on the belly and adjacent belly-skin as illustrated in Fig. 8, *BM*. The back-muscle transplant is omitted in the figure. Lettering the same as in Fig. 6.

FIG. 8. Appearance of tail-skin grafts on the belly under which muscle transplants had been inserted four days previous. *TM*, graft (under which tail-muscle transplant had been inserted), showing decided macroscopic signs of histolysis; *BM*, graft (under which back-muscle transplant had been inserted), showing only slight macroscopic signs of histolysis; *BS*, belly skin.

FIG. 9. The same grafts as illustrated in Fig. 8 eleven days later. Note excessive reduction in size of graft over tail-muscle transplant as compared with size of graft over back-muscle transplant.

PLATE I



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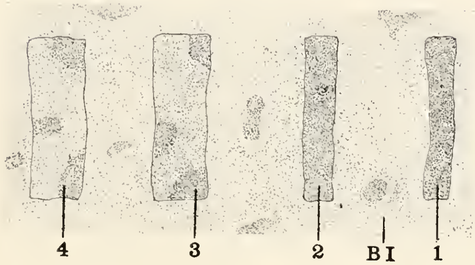

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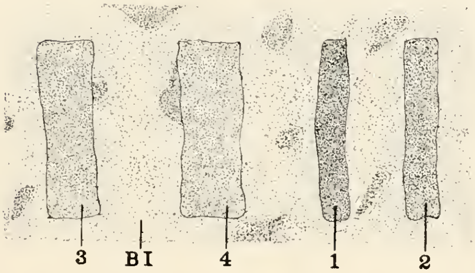

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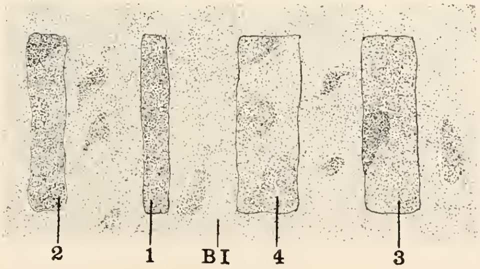

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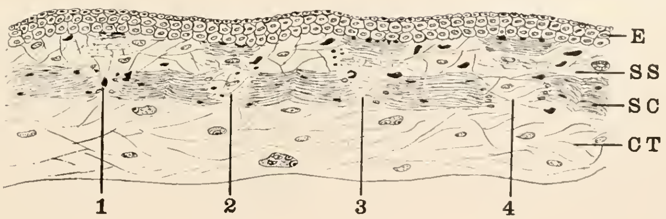
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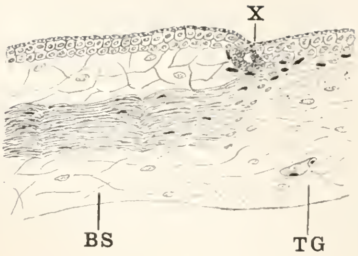

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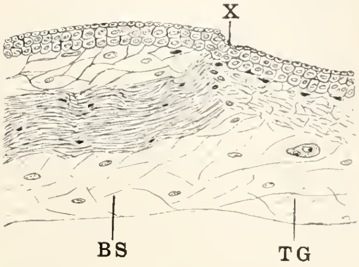

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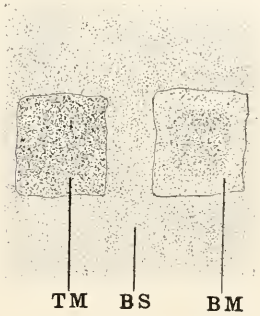

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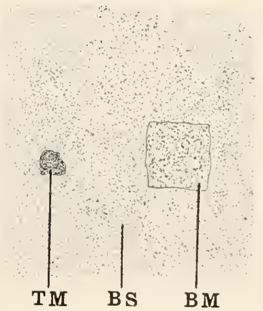

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The four back-skin grafts transplanted to the tail proved to be remarkably resistant to histolysis during the early stages of tail atrophy. This confirms the findings of Lindeman (1929). All of the four back-skin transplants appeared to be equally resistant, but it was noted that as more pronounced atrophy of the tail ensued (see Table II, 16-day observation), the grafts that had been transplanted to the more anterior regions of the tail underwent a certain degree of histolysis, while those previously placed on wound area number 4 never gave evidence of degeneration. It would appear, therefore, that the comparatively greater rate of muscular histolysis in the anterior regions of the tail is capable, eventually, of inducing even back skin to degenerate.

3. *Effect of Atrophying Muscle on Histolysis of Tail Integument*

It was shown by Helff (1926) that atrophying tail muscle is capable of inducing histolysis in opercular skin grafts when transplanted beneath the latter. More recently Helff and Clausen (1929) have shown that tail muscle grafts undergo approximately a 62 per cent greater atrophy during metamorphosis when transplanted to the back than is true of back muscle transplanted from one side of the back to the other. It therefore seemed likely that the normally atrophying musculature of the tail may function to facilitate or even to accelerate the histolysis of tail integument during involution. Experiments were consequently devised to test out this assumption.

The operations performed were briefly as follows: Two grafts of integument (4 mm. square) were removed one from each side of the middle region of the tail of normal larvæ. These were then transplanted, autoplastically, one on either side of the mid-ventral line of the belly and the grafts allowed to heal firmly to the surrounding integument. Three days later a cube of back muscle (1.5 mm. square) was removed from the same larva and transplanted beneath one of the integumentary grafts. At the same time a similar sized piece of tail muscle was removed from the same larva and inserted beneath the other integumentary graft. Forty larvæ were so operated on in all. The larvæ were now allowed to metamorphose in order that the effects of the two types of muscle transplants on the tail-skin grafts could be determined.

The first signs of normal tail atrophy of the operated larvæ were evident within one or two days following the transplantation of the integumentary and muscle grafts. Usually about three days following the operative procedure, distinct macroscopic signs of histolysis were

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evident in the tail-skin graft underneath which tail muscle had been inserted. This consisted chiefly in a characteristic darkening of the entire surface of the integument (*TM*, Fig. 8, Plate 1). The other skin graft, underneath which back muscle had been inserted, appeared more normal, although histolysis as evidenced by a darker coloration of the skin was typical of a central area of the transplant directly over the muscle graft (*BM*, Fig. 8, Plate 1). Histological sections made at this stage through these two tail-skin grafts (Figs. 6 and 7, Plate 1) gave evidence that more pronounced signs of tissue disintegration were evident in the graft over the tail-muscle as compared with the graft over the back-muscle transplant. Following this period, both integumentary grafts underwent a progressive histolysis and consequent reduction in size. Fifteen days following transplantation, the relative appearances of the two grafts were approximately as represented in Fig. 9, Plate 1. By the time the integumentary graft in association with tail muscle had practically been obliterated, the other graft associated with back muscle was approximately but 50 per cent reduced in area. The results serve to emphasize, therefore, that atrophying tail muscle is decidedly more effective in hastening the histolysis of tail integument than is the case of atrophying back muscle. The further inference may be drawn that the musculature of the tail is normally favorable towards or may even accelerate the histolysis of the integument of the latter organ during metamorphosis.

Discussion

The argument might well be advanced that one would expect a highly differentiated tissue like muscle to undergo perhaps complete degeneration following transplantation even in normal non-metamorphosing larvæ. This, however, cannot be accepted as a possible explanation of the present results since Helff and Clausen (1929) transplanted various types of anuran muscle in non-metamorphosing larvæ and recorded an average reduction in volume of less than ten per cent over a period of three weeks. The reduction in volume of the muscle transplants as described in the present paper must therefore be considered due mainly to the operation of definite histolytic factors functional during larval involution.

The question of whether or not the atrophying musculature of the tail actually tends to accelerate the integumentary histolysis of the latter organ is an interesting one. The results of the present paper confirm those of Helff (1926) in that atrophying tail muscle is capable of inducing histolysis of certain integuments. The results of Lindeman (1929), regarding the resistance of back skin to histolysis when transplanted to the tail and the total histolysis of tail-skin transplanted to the back, are also corroborated. Slight histolysis, it would seem, may be induced in back-skin grafts when transplanted to the anterior regions of the tail for a considerable period of time. Lindeman's transplants, it may be stated, were all made to the central or more posterior tail regions and hence corresponded to the results of the present work in which skin grafts had been transplanted to areas 3 or 4 as represented in text Fig. 2. The results of tail- and back-muscle transplantation beneath tail-skin grafts gave distinct evidence that although the integument of the tail is capable of undergoing complete histolysis during metamorphosis when transplanted to the back, its rate of histolysis is probably accelerated considerably when in contact with the atrophying musculature of the tail.

The results adequately support the contentions of Helff (1928), Helff and Clausen (1929), and Lindeman (1929) in that the growth of the urostyle cannot be looked upon as the fundamental cause of anuran tail atrophy, but that the various tissues of the tail are hereditarily specific and susceptible to histolytic agents probably transported through the blood stream. This latter generalization would seem to be particularly true for the integumentary and muscular elements of the larval structure. The present results finally indicate that the integument and musculature are not equally susceptible to histolysis at all levels of the tail, but conversely, appear to exhibit what might be called a susceptibility gradient in that anterior muscular and integumentary regions are more susceptible to histolysis than is true of more posterior regions. Granting that the present results have proved the existence of a susceptibility gradient to histolysis, it is of interest to inquire regarding the origin of this gradient. The different degrees of susceptibility must either be inherited during the embryonic formation and development of the various tissues of the tail or else acquired during the growth period of the larval organ. To date, there are no experimental results available to warrant an adequate explanation of this point.

SUMMARY AND CONCLUSIONS

Preliminary observations indicated that different regions of the larval anuran's tail undergo histolysis at different speeds.

1. Uniform-sized, tail-muscle grafts were secured from extreme anterior and posterior regions and from two intermediate areas and transplanted, autoplastically, to the backs of *Rana pipiens*' larvæ. During metamorphosis, anterior grafts invariably histolyzed with greater rapidity (10 to 25 per cent) as compared with posterior muscle grafts. Intermediate muscle grafts histolyzed at proportionate speeds.

2. Similar grafts of tail skin were autoplastically transplanted. Regardless of their location on the back, skin grafts from anterior tail regions always underwent histolysis at a greater speed and prior to grafts from more posterior regions. Total histolysis of anterior grafts was frequently observed at a time when posterior grafts presented less than 50 per cent disintegration.

3. The comparative histolytic influence of tail and back muscle was questioned by the transplantation of muscle beneath integumentary grafts previously made to the belly. Integumentary grafts over tail-muscle transplants invariably underwent approximately at least 50 per cent greater histolysis as compared with grafts over back-muscle transplants.

4. The conclusion is reached that anterior regions of the larval anuran's tail undergo histolysis with a greater speed and prior to more posterior regions. A definite anterio-posterior gradient may be said to exist in this regard, the cause of which, however, is still undetermined.

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THE INNERVATION OF THE HEART OF THE ELASMOBRANCH, *SCYLLIUM CANICULA*

BRENTON R. LUTZ

(From the Zoölogical Station, Naples, and the Physiological Laboratory of Boston University School of Medicine)

The elasmobranch fishes have certain features, both structural and physiological, which make them of interest from a comparative viewpoint. The well developed cranial parasympathetic system and the poorly developed sympathetic pointed out by Müller and Liljestrand (1918), the abundant chromaphil tissue quite separate from the interrenal tissue first described by Balfour (1878), the extraordinarily high urea content of the blood, the significance of which Baglioni (1906) has studied, the marked cardiac and respiratory inhibition and the absence of vaso-motor responses noted by Schoenlein and Willem (1894) and the inhibitory action of adrenaline on the heart first noted by Macdonald (1925) are some examples. An attempt, however, to find a detailed description of the vagus supply to the heart of *Scyllium canicula*, which has been the chief type used, was unsuccessful, although the course of the vagus in certain fishes has been described by Dogiel and Archangelsky (1906). In the pike (*Esox lucino*) these authors trace a ramus cardiacus from the visceral trunk to the sinus-auricle junction where a plexus is formed giving off fibers to the ventricle. In *Scyllium*, Marshall and Hurst (1905) state that the visceral branch of the vagus gives off cardiac nerves to the heart. In the dogfish, *Squalus acanthias*, Norris and Hughes (1920) describe the cardiac ramus as arising from the post-branchial branch of the fourth branchial division of the vagus (fifth branchial nerve). In an investigation of the cardio-inhibitory reflex and of the visceral afferent nervous pathway in *Scyllium* the writer found it necessary to examine the vagus supply to the heart in some detail. Incidentally some observations were made on the presence of vagal tone.

Bottazzi (1902) attempted to demonstrate an accelerator innervation of the heart of *Scyllium*, with the medulla separated from the spinal cord, by stimulating various sections of the cord and the first sympathetic ganglion, but obtained no acceleration in the rate. With the idea that possibly there were accelerator fibers entering with the vagus, he stimulated this nerve electrically with gradually increasing strengths of stimulus, but got only inhibition. He did not perfuse the gills during the procedure. Müller and Liljestrand (1918), in a morphological study, could find no nerve fibers running to the heart from the first large sympathetic ganglion in elasmobranchs. Since Zwaardemaker (1925) claims to have found cardio-accelerator nerves in *Petromyzon* in which previously both Greene (1902) and Carlson (1904) had failed to demonstrate such nerves, the writer considered it worth while to repeat, with perfusion of the gills, Bottazzi's experiments, and to try certain other procedures which might possibly reveal the presence of an accelerator control of the heart.

Material and Methods

Specimens of *Scyllium canicula* averaging 300 grams were used. Gross dissections were made in living fishes with the brain destroyed anterior to the optic lobes and the spinal cord pithed posteriorly from various levels. Morphological observations were checked by stimulating the nerves in question both centrally and peripherally with a faradic current, or by cutting all nerves except the one to be examined and testing its function in reflex cardio-inhibition. In one case a complete dissection was stained with osmic acid. By a method described in a previous paper (Lutz, 1930) the heart and the respiratory rate were recorded.

A similar type of preparation, with certain parts of the nervous system destroyed and with the gills perfused, was used to investigate the possible existence of accelerator influence.

Results

In studying reflex cardio-inhibition it was at once obvious that cutting the cardiac root of the visceral branch of both vagi failed to prevent reflex cardiac arrest on stimulation of the skin and various other points. On the assumption that *Scyllium* might be similar to *Squalus* with respect to vagus innervation, the post-branchial root of the fourth branchial division (fifth branchial nerve) on each side was also cut. This procedure prevented the elicitation of reflex cardio-inhibition. Cutting either pair of roots alone did not prevent direct inhibition of the heart on stimulation of the nerve anterior to the fourth branchial division. All four nerves must be cut, or section of the main trunk be made anterior to the fourth branchial division in order to remove all vagus influence. A careful dissection was then made of many specimens and the two branches from each vagus were found to run to the Cuverian duct and there anastomose on the wall of this large vein before passing to the heart. Weak faradic stimulation of the cut distal portion of each branch produced inhibition, and stimulation of the central end caused reflex cardio-inhibition provided any one of the branches remained intact.

Reflex cardiac and respiratory inhibition was obtained on mechanical stimulation of the dorsal surface of the ventricle either by pinching it lightly with the fine forceps or by sticking the epicardium with a sharp needle (Fig. 1, *A* and *B*). This was also obtained after the aorta had been transected. Reflex cardio-inhibition was also elicited on weak faradic stimulation of the ventricle in a fish with the spinal cord pithed posteriorly from the fifth vertebra and the hypobranchial nerve and all other spinal nerves of the region cut. All branches of the vagus except the post-branchial ramus of the fourth branchial division on each side were cut (Fig. 1, *C*). The cardio-inhibition was accompanied by reflex movements in the anterior gill region, apparently through the more anterior cranial nerves. It therefore appears that the vagus innervation of the heart, both efferent and afferent, is by way of two pairs of branches, one pair from the post-branchial ramus of the

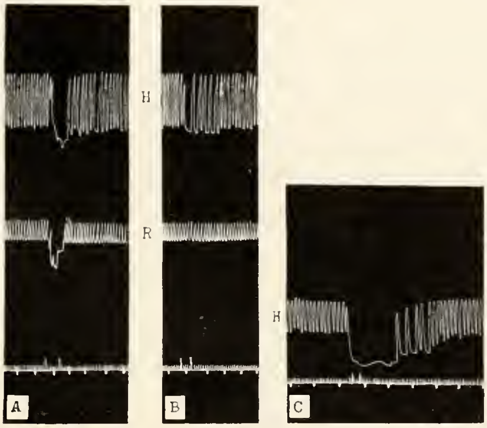

FIG. 1. Reflex cardiac and respiratory inhibition on stimulation of the dorsal surface of the ventricle of *Scyllium canicula* with the forebrain destroyed and the spinal cord pithed posteriorly from the fifth vertebra. The upper tracing is the heart record; the middle is respiration. The large intervals on the time-signal record are ten seconds. *A*, the effect of sticking several times with a sharp needle. *B*, the needle was inserted gently just beneath the epicardium. *C*, weak faradic stimulation of the ventricle after all branches of the vagi were cut except the post-branchial ramus of the fourth branchial division on each side. The hypobranchial and other spinal nerves from the intact portion of the cord were also cut.

fourth branchial division, as in *Squalus,* and the other pair from the visceral branch of the vagus.

Cadiat (1879) showed that double vagotomy in *Scyllium* results in an increased rate of the heart. McWilliam (1885) and Kolff (1908) have found the same result in teleosts. The writer found a similar effect (Table I). In some instances cutting one vagus gave a considerable increase in rate which was further augmented when the remaining vagus was cut. The effect is most strikingly seen when the initial rate is low. Such figures have been taken to mean that the vagus normally exerts a tonic influence on the heart. In view of the great ease with which reflex inhibition of the heart occurs in elasmobranchs, as shown by Schoenlein and Willem (1894), Lyon (1926), and Lutz (1929), it is doubtful whether the result of vagotomy under the usual experimental conditions is an indication of the existence of normal vagal tone. In one case the initial rate, some time after exposing the heart, was 30 per minute. After cutting the previously prepared left vagus the rate was 34. Following an hour of experimentation, consisting of producing reflex inhibition by various means, the rate fell to 12. Then cutting the right vagus resulted in an increase to 30. Even gentle blowing on a wound is sufficient to produce a considerable reflex slowing of the heart.

Table I

The effect of vagotomy on the rate of the heart of Scyllium canicula, showing an apparent release from vagal tone.

Before vagotomy *Beats per minute*	After double vagotomy	Before vagotomy *Beats per minute*	After cutting one vagus	After cutting second vagus
30	44	16	33	42
24	40	36	36	38
22	36	26	32	36
18	32	32	32	40
20	34	24	24	38
36	44	39	42	44

Many attempts were made to obtain acceleration of the heart by stimulating the medulla or the cord with the vagi cut and with the gills perfused, but, confirming Bottazzi (1902), no acceleration occurred. The vagus was cut high, or in some cases the medulla itself was destroyed, in order not to interfere with any possible union of sympathetic fibers from the hypobranchial or other roots of spinal nerves with the vagus, although a sympathetic supply from these sources was considered quite unlikely for morphological reasons. The cord was pithed posterior to the eleventh vertebra and a section three centimeters long was exposed anterior to this vertebra. In *Squalus* this section of the cord (fifth to twelfth vertebra) gives rise to fibers passing to the first sympathetic ganglion, according to Müller and Liljestrand (1918). Faradic stimulation of either the exposed cord or the anterior cut surface of the medulla or mid-brain did not produce acceleration of the heart. Neither did faradic stimulation of the first large sympathetic ganglion accelerate the heart. In this case any possible accelerator pathway from the ganglion to the heart by way of the cardiac ramus of the vagus was not disturbed.

Since there is some evidence that the vagus center normally has a tonic inhibitory effect on the heart, and since a period of acceleration is sometimes seen following a period of inhibition, it was considered that during activity of the cardio-inhibitory center there might be a lowering of tone in the accelerator center. After a period of increased activity of the vagus center its tone, through fatigue, might be lowered and this with the return of the accelerator center to its former condition might account for the acceleration sometimes seen. With the vagi cut and the vagus center active a lowering of accelerator tone would be expected to result in slowing of the heart. To test this a preparation with the medulla and cord to the twelfth vertebra intact was used with the gills perfused. Various means shown previously (Lutz, 1929) to produce marked reflex cardio-inhibition were used, such as faradic stimulation of the central end of the cut vagus or the cut lateral line nerve, vigorous mechanical stimulation of the nasal openings, or pinching the sides of the fish between heavy forceps. The records of the heart rate during such procedures did not show slowing, and consequently it is concluded that no lowering of tone in an accelerator center occurred. This can not be taken as complete proof, however, that no accelerator center exists, but since, in addition, no accelerator fibers have been demonstrated, it points strongly to its absence. Acceleration of the heart sometimes seen after a period of inhibition is probably due to fatigue of the inhibitory center.

I wish to thank Dr. Reinhard Dohrn and Dr. Enrico Sereni for the many courtesies extended at the Zoölogical Station, Naples, and Edna B. Lutz for technical assistance.

SUMMARY

1. Two branches of the vagus go to the heart of *Scyllium canicula,* each carrying afferent and efferent fibers, one from the post-branchial

ramus of the fourth branchial division (fifth branchial nerve), and the other from the main visceral trunk.

2. No acceleration of the rate of the heart occurred on faradic stimulation of the medulla, spinal cord, or first large sympathetic ganglion with the vagi cut.

3. No slowing of the rate of the heart occurred, due to lowering of tone in an accelerator center, on reflex excitation of the cardio-inhibitory center with the vagi cut.

4. Both morphological and physiological evidence indicate the lack of accelerator fibers to the heart of the elasmobranch.

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THE VISCERAL AFFERENT PATHWAY IN THE ELASMOBRANCH, *SCYLLIUM CANICULA*

BRENTON R. LUTZ

(*From the Zoölogical Station, Naples and the Physiological Laboratory of Boston University School of Medicine*)

With the elaborate morphological development of the autonomic nervous system in the higher vertebrates, the efferent innervation of the abdominal viscera has tended toward physiological differentiation. However, while the orthodox view holds that the cranial parasympathetic carries the excitatory fibers for the stomach and intestines, and the sympathetic contains the inhibitory fibers, Langley (1898) found inhibitory fibers to the stomach in the vagus of the rabbit, Morat (1893) found excitatory fibers to the stomach and intestine in the splanchnic of the dog, and Carlson, Boyd and Pearcy (1922) have found that both splanchnics and vagi of the cat carry both kinds of fibers to the stomach. Even in the elasmobranch fishes, where the autonomic nervous system is still morphologically simple, the two motor functions are not distinctly separated. Bottazzi (1901) and Müller and Liljestrand (1918) have reported motor activity of the stomach on stimulation of either the vagus or the first sympathetic ganglion, and the latter workers report also inhibitory bundles in the vagus.

On the afferent side of visceral innervation there is ample evidence in the higher vertebrates that there are fibers running from the abdominal viscera to the cord by way of the autonomic system (Head, 1893; Ranson and Billingsley, 1918). Stimulation of the stomach causes acceleration of the heart, according to Dmitrenko (Ranson, 1921), through afferent fibers mainly in the splanchnic nerve, but to some extent in the vagus. Brodie and Russell (1900) obtained slowing of the heart on stimulation of the central ends of the vagi to the stomach and concluded that afferent impulses are carried up the vagi as well as along the splanchnics in the cat.

In the elasmobranch fishes one might expect the vagi to take the larger part in carrying afferent fibers from the abdominal viscera although the writer found in the literature no reference to the vagus as an afferent pathway from these organs. Lyon (1926) noted cardio-inhibition on stimulating the stomach and other abdominal organs in sand sharks (*Carcharias*), but reported no attempt to locate the afferent pathway. In a teleostean fish (eel) McWilliam (1885) could obtain no cardio-inhibition on stimulation of the abdominal organs, and while stimulation of the central end of the vagus on the oesophagus caused a marked cardio-inhibitory response, stimulation of either vagus after it had passed to the stomach had no effect. Mills (1886), however, working on the teleost, *Batrachus tau*, never failed to get reflex cardio-inhibition from the stomach and intestine, but he made no examination of the nervous pathways involved.

MATERIALS AND METHOD

The method of operating on *Scyllium canicula* out of water, and of recording the heart and respiratory rates has been described in a previous paper (Lutz, 1930). The spinal cord was transected at a desired level and pithed posteriorly. The gills were perfused through the mouth and respiration quickly became regular. The vagus and its branches were exposed through the anterior cardinal sinuses. The first large sympathetic ganglion and accessory ganglia were exposed through the posterior cardinal sinuses. Both operations were, of course, accompanied by unavoidable profuse bleeding, but since the writer found that the medullary centers of *Scyllium* would continue to be reflexly functional for about one hour after aortic transection, the bleeding did not interfere.

RESULTS

Mechanical and faradic stimulation of the stomach, spiral valve, or mesentery caused cardiac and respiratory inhibition when the spinal cord was destroyed posteriorly from the first vertebra, but not when the vagi were cut either immediately posterior to the origin of the fourth branchial division (fifth branchial nerve) or just posterior to the origin of the cardiac rami from the visceral branches (Figs. 1 and 2, *A*, *B* and *C*). With the medulla, cord to the fourteenth vertebra, and cardiac vagi intact neither cardiac nor respiratory inhibition could be elicited on faradic stimulation of the first large sympathetic ganglion, but the same stimulus applied to the parietal peritoneum 3 mm. from the ganglion or to the adjacent oesophageal wall produced marked cardiac diastolic inhibition and sometimes respiratory inhibition as well (Fig. 2, *E* and *F*). Removal of the large sympathetic ganglion on each side and several accessory ganglia posterior to it or destruction of the spinal cord as high as the first vertebra failed to prevent elicitation of the inhibitory responses on stimulation of the stomach, spiral valve, or mesentery so long as the visceral branches of the vagi remained intact. Faradic stimulation of the central end of either visceral branch of the vagus, cut at the stomach, also produced cardio-inhibition. Vigorous mechanical or faradic stimulation of the liver and the testis failed to evoke either cardio-inhibition or respiratory slowing even with the entire cord intact. Stimulation of the kidney, epididymis, ovary, and uterus were effective in producing both reflexes when the cord was not destroyed (Fig. 3). Stimulation of the kidney was without effect with the cord pithed posterior to the third vertebra. No attempt was made to locate the portion of the cord receiving the afferent fibers, and no examination

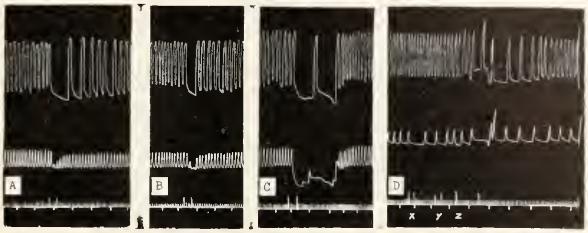

FIG. 1. Reflex cardiac and respiratory responses on stimulation of the abdominal viscera. Forebrain and cord from the third vertebra destroyed. Upper record, heart; middle, respiration. The large divisions on the time-signal record are ten seconds. *A*, spiral valve pinched with forceps. *B*, stomach pinched. *C*, gentle blowing on the viscera. *D*, the vagi have been cut below the cardiac rami. *x*, spiral valve pinched. *y*, stomach pinched. *z*, points of the forceps inserted in the nasal openings.

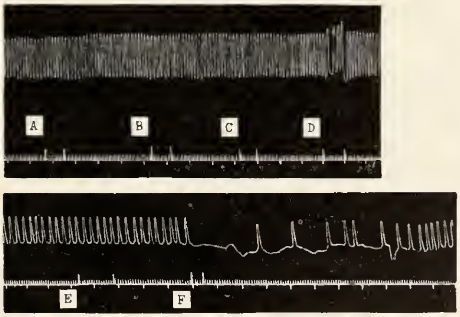

FIG. 2. Reflex cardiac inhibition. Forebrain and cord from the thirteenth vertebra destroyed. Vagi cut below the cardiac rami. *A*, stomach pinched with forceps. *B*, faradic stimulation of the stomach. *C*, spiral valve pinched. *D*, forceps inserted in the nasal openings. *E*, faradic stimulation of the first large sympathetic ganglion. *F*, faradic stimulation of the peritoneum 5 mm. from the ganglion.

of the relation of the vagus to the kidney, epididymis, ovary, and uterus was made.

The results presented above indicate that in the elasmobranch, *Scyllium canicula*, the vagus carries afferent fibers from the stomach, spiral valve, and mesentery. In view of the fact that sensory stimulation at a great variety of points produces reflex cardiac and respiratory inhibition in *Scyllium* (Lutz, 1929), the failure of stimulation of the first large splanchnic ganglion to elicit these responses may be taken to indicate a lack of afferent fibers through this ganglion. The liver and testis also have no afferent fibers, stimulation of which gives either cardiac or respiratory response. Lyon (1926) likewise obtained no cardio-inhibition on stimulation of the liver in sand sharks (*Carcharias*).

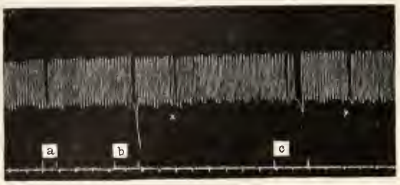

FIG. 3. Reflex cardiac inhibition. Entire cord intact. *a*, uterus pinched with forceps. *b* and *c*, ovary pinched. *x*, viscera handled. *y*, ovary handled.

SUMMARY

1. The vagus in the elasmobranch, *Scyllium canicula*, is an afferent pathway from the stomach, spiral valve, and mesentery.
2. There are no afferent fibers passing through the first large sympathetic ganglion or from the liver and testis, stimulation of which produces either cardiac or respiratory inhibition.
3. Removal of the sympathetic ganglia or destruction of the spinal cord as high as the first vertebra fails to prevent elicitation of the inhibitory reflexes provided the visceral branches of the vagi remain intact.
4. Faradic stimulation of the central end of a visceral branch of the vagus at the stomach produces cardiac and respiratory inhibition.

I wish to thank Dr. Reinhard Dohrn and Dr. Enrico Sereni for the many courtesies extended at the Zoölogical Station, Naples, and Edna B. Lutz for technical assistance.

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NOTES ON A FRESH-WATER MEDUSA FOUND IN STALLWORTH LAKE, TUSCALOOSA, ALABAMA

WILLIAM E. WHITE

(From the Department of Zoölogy, University of Alabama, and the Department of Biology, New York University.)

During a series of collecting trips, fresh-water medusæ were found in Stallworth Lake near Tuscaloosa, Alabama (White, 1929). They were first observed on September 14, 1928. I visited the lake at least twice a week and never failed to find medusæ until the ninth of October. They did not reappear after this date, although I continued to visit the lake at regular intervals. The approach of cold weather does not seem to have caused their disappearance, as has been suggested in other cases, since warm weather persisted for several weeks after they were last seen. Specimens, which I had placed in large jars of pond water in the laboratory, lived for about two weeks longer, gradually wasting away in size and finally dying. The water contained small Crustacea, so it is hardly possible that lack of food caused their death.

The medusæ were sensitive to changes in intensity of light and to disturbances in the water. In general they were more abundant at the surface on bright days than on cloudy ones. Although I could not bring the medusæ to the surface of the lake at night by shining a flashlight on the water, they became very active when the lights were suddenly turned on in a dark laboratory. I have brought them to the surface by violently thrashing the water at a considerable depth with a long board or iron pipe. They also became very active when the water of the aquarium was stirred.

No hydroid stages were found although repeated searches were made for them from time to time during the fall of 1928 and the spring of 1929. Quantities of scrapings from the piles of the pier and the surfaces of submerged boards and rocks were examined. The hydroid stage was probably present, however, and I believe that if a more extensive search had been made, polyps would have been found in considerable numbers, since the medusæ were so abundant. Perhaps the hydroids were confined to a few localized areas, which would account for my not finding them.

The medusæ have not been observed in the lake since October, 1928. I examined the lake at frequent intervals during the entire summer of 1929 but did not see a single medusa.

THE LAKE

Stallworth Lake is situated on a terrace midway between the business district of Tuscaloosa, Alabama, and the Warrior River. It was formed in 1918 by damming up four acres of marshy land and is supplied by springs which keep a small stream running from the lake. This streamlet flows into a small pond which finally empties into the river. The depth of the lake varies from a few inches to about twenty feet. Along the edges the bottom is sandy, but in the deepest parts it is covered with mud. There is an abundance of plant life in the lake. Fresh-water algæ give the water a greenish color. Several species of the higher plants are also present. Willows line the dam on one side of the lake. The fauna of the lake is abundant and varied. Numerous species of Protozoa and members of the other invertebrate phyla were constantly encountered during the search which was undertaken for the hydroid. Fish have been introduced from hatcheries in Mississippi and the Warrior River. A large number of turtles are found in the lake, no doubt coming in from the river. Mussels of the genus *Lampsilis* abound in the lake as do snails of the genus *Physa*. In the spring and early summer the lake is used as a swimming pool. Later it is little frequented due to the rather slow change of water. There is a pier, extending out over the water from one shore, which connects the boat house with a small island. A part of this pier is covered by a roof. Most of the specimens were collected from the water on the sides of this pier.

Although the Warrior River is subject to sudden rises which spread it over the surrounding territory, the level of the river has never reached that of the lake. The lake has never been drained, but every year the water is lowered about two feet to facilitate cleaning and repairing. The pH of the water at the time the medusæ were found was 7.2. As for the temperature of the lake, it never freezes, and in the summer the surface may reach a temperature of eighty-five degrees Fahrenheit, due to the very slow turnover of the water.

HISTORICAL SURVEY OF THE FRESH-WATER MEDUSÆ

Although marine medusæ have been known for centuries (Aristotle mentions several species), a fresh-water medusa was not reported until 1880. In June of that year Mr. Sowerby, secretary of the Royal Botanical Society, found medusæ in the Society's *Victoria regia* tank in Regent's Park, London. He gave specimens to Lankester and Allman who studied and described them. Allman proposed the name *Limnocodium victoria* for the new form, while Lankester called it *Craspedacusta sowerbii*, in allusion to the relation of the otocysts to the velum. For a number of years the medusa was known as *Limnocodium sowerbii*, which evidently violates the accepted rules of nomenclature. An attempt was made to have this name validated, but the petition was denied by the International Commission on Zoölogical Nomenclature (Mayer, 1910). Lankester's name, *Craspedacusta sowerbii*, published on June 17, 1880, has priority, and is clearly the correct one. I call attention to this point since the name *Limnocodium* has appeared in the literature within the last two years. *Craspedacusta sowerbii* appeared in the lily-tank of Regent's Park for a number of years, finally disappearing in 1893. Romanes (1880) reported some interesting experiments on the physiology of this fresh-water medusa. Bourne (1884) first described the hydroid stage. Fowler (1890) described medusoid bud formation and gave a complete bibliography of the literature up to that time. Günther (1894) worked out the histology of the medusa stage of this form.

In the last half-century fresh-water medusæ have been reported many times from widely separated localities. Edward Potts (1897) reported the first fresh-water species from America. He had already found the hydroid stage of this form (1885) and had called it *Microhydra ryderi*. Ryder (1885), believing that *Microhydra ryderi* was probably the hydroid stage of a medusa and that this medusa would prove to be generically different from *Craspedacusta*, because of certain differences between *Microhydra ryderi* and the hydroid stage of *C. sowerbii*, proposed the generic name Pottsia, should the medusa stage be found. Why he should have favored separate names for the hydroid and the medusa is not clear. At any rate the name was not valid and was never used. Payne (1924) has demonstrated that *Microhydra ryderi* is a species of the genus *Craspedacusta*, and has designated it *Craspedacusta ryderi*, abolishing the genus *Microhydra*. He reported the complete life cycle of this species (1926).

Although the evidence is not conclusive and only a study of the development could permit a final decision, presumably, the medusæ found by Hargitt (1907), Coker (Payne, 1924), and Garman (1916), and assigned to the European species *Craspedacusta sowerbii*, are specifically identical with the forms found in Boss Lake, Indiana (Payne, 1924) and in Stallworth Lake, Alabama, and should be referred to the American species, *Craspedacusta ryderi*.

Roch (1924) found fresh-water medusæ in a mill stream near Berlin. The largest specimens measured 0.68 millimeter in diameter. They differed from the young medusa of *Craspedacusta ryderi* (then called *Microhydra ryderi*) in having sixteen tentacles which were not of uniform length and which did not appear simultaneously in the course of the development of the medusa. He also stated that the great geographical separation of this form from *Microhydra ryderi* spoke little for their identity. To this form he gave the name *Microhydra germanica*. It is, no doubt, a member of the genus *Craspedacusta*, and whether it is a different species from *Craspedacusta sowerbii*, the European form, can only be determined by a study of the hydroid, budding, and medusoid bud formation. If, indeed, it is a different species, the correct name would be *Craspedacusta germanica*.

Other species of fresh-water medusæ which have been reported up to the present time are *Limnocnida tanganjicæ* (Günther, 1893) from Africa, *Craspedacusta kawaii* (Oka, 1908) from China, and *Limnocnida indica* (Annandale, 1912) from India. Payne (1924, 1926) gives an extensive bibliography covering fresh-water medusæ to that date. Other papers not listed are cited here. These include a report by Goette (1908), who found them in Strassburg. Pelosse (1919) found them in a park in Lyon. Backhoff (1924) reported them from Stettin. Flower and Lockyer (1928) reported the reappearance of *Craspedacusta sowerbii* in the Royal Botanic Society's garden in Regent's Park, London, where fresh-water medusæ were first reported in 1880. Rupert Vallentin (1930) found medusæ in the Exeter ship canal in July of 1928 and 1929.

The Adult Medusa

Payne (1924) has given an excellent description of the medusa of *Craspedacusta ryderi*. Since the medusæ of Stallworth Lake conform very closely to his description, I shall give here merely a few details wherein they differ from those found in Boss Lake. Payne found that the sexes were so alike that the only way to tell them apart was by an examination of the gonads. I found only males, although a number of the medusæ were examined by smear and sectioning methods. I am inclined to think that if females were present, at all, they occurred in very small numbers. As I did not arrive in Tuscaloosa until early fall, all the medusæ were fairly large—none measuring less than fourteen millimeters in diameter. The medusæ are, evidently, slightly larger than those found by Payne, the largest specimens measuring about twenty millimeters in diameter, when in the relaxed condition. There is a correspondingly greater number of tentacles—over four hundred were counted on each of several individuals. Although the tentacles of *Craspedacusta* have, heretofore, been placed in three size groups, they seem to fall into four fairly distinct sets. Allman (1880) shows this clearly in his drawing, but uses only three groups in his description. First, there are the perradials, four large tentacles lying at the terminations of the radial canals. Those of the next group are almost as large and lie between the perradials. There are about twenty-five of these. The third set is considerably smaller than the first two. There are about thirty-five in this group, which are scattered among the larger tentacles. The smallest tentacles form a hairlike fringe around the periphery of the umbrella. They are so numerous that they are difficult to count. Three hundred and thirty-eight were counted on one individual. Payne (1924) found sixty-nine lithocysts in a specimen five millimeters in diameter. I have counted over two hundred in several mature specimens.

These differences may have been caused by developmental differences or by dissimiliar environmental conditions. In other respects the medusæ from the two localities are quite similar. The study of the hydroid, if it is found, and the developmental processes may, possibly, bring out specific differences between the Alabama and Indiana forms, but at the present time I am inclined to think that the medusæ are specifically identical and have tentatively assigned the form from Stallworth Lake to the species *Craspedacusta ryderi.*

SPERMATOGENESIS

When the medusæ were first observed specimens were taken to the laboratory. The gonads of a number of these were excised and fixed in Bouin's fluid. Sections of these gonads were cut at five microns and stained with iron hæmatoxylin. Others were mordanted in Flemming's fixative and stained with Flemming's triple stain. These sections demonstrated that all of the medusæ examined were males. In spite of the fact that all of the medusæ were fully grown, various stages of spermatogenesis were observed in each section. Since no detailed study of spermatogenesis in a fresh-water medusa has been reported, certain observations will be given here.

From the subumbrella side of each radial canal there projects a long sac-like pouch, lined by a layer of entodermal epithelium, a single cell in thickness, which is continuous with that of the radial canal and which forms the cavity of the gonad. The mesogloea is much reduced in the gonad, forming only a very thin layer at the base of the entoderm cells. The rest of the wall of the gonad is thick and composed of germ cells and developing sperm, with a thin superficial covering of ectodermal tissue. The entoderm cells are columnar in shape with large irregular vacuoles in their cytoplasm. The nuclei are found in the end of the cells distal to the cavity of the gonad. They contain large centrally placed nucleoli. Adjacent to this entodermal layer are the spermatogonia. Since only mature gonads were secured, no information can be given concerning the origin of the germ cells. Günther (1894) considered them to be of ectodermal origin. He distinguished sperm mother cells (spermatogonia), daughter spermatoblasts (secondary spermatocytes), spermatids, and spermatozoa, but found them to be too small for any accurate observations. The spermatogonia lie nearest the entoderm, while the later stages occur further toward the outside of the gonad. The spermatozoa are found just under the layer of ectoderm. They are, presumably, shed by rupturing this thin superficial layer.

The spermatogonia form a closely packed layer nearest the entoderm. They are irregular cells, having a diameter of about six microns. The nuclei are large, filling a considerable part of the total volume of the cell. They have large deeply staining nucleoli, which are usually centrally placed. The nucleolus is generally single, although double ones occur. The nuclear membrane is delicate but can be readily seen under 1.5 oil immersion lens. The scarcity of mitotic figures among the spermatogonia indicates that division of these stages had stopped in the gonads under observation. The condition of the chromatin, packed into a single large nucleolus, seems to be the typical resting stage of the spermatogonia. Figure 1 shows a resting spermatogonium.

The first indication of the change from spermatogonia to primary spermatocytes is a fragmenting of the large nucleolus. It may break into several pieces which gradually diminish in size. In a number of cases, however, the nucleolus breaks into two portions which move apart within the nucleus before fragmentation begins. When this process does start, one of the pieces disappears before the other, leaving for a time a large dark staining body at one side of the nucleus and finely granular chromatin at the other. As fragmentation of the nucleolus continues the chromatin becomes finely granular and is practically unstainable with iron hæmatoxylin. It stains with safranin, however, in this condition. In cells adjacent to those in the granular stage, leptotene threads begin to appear. The chromatin at this stage forms knots which are suspended just under the nuclear membrane. The nucleus has attained its maximum size at this time. From this irregular network heavy threads develop. There is evidently an increase in the total quantity of chromatin, certainly in the stainable part, since the amount in the heavy syndesis mass shown in Fig. 6 is much greater than the delicate mesh shown in Fig. 5. The nucleus is not as large at this stage as it was in the preceding one.

The next change which can be observed is the disappearance of the nuclear membrane and a definite flattening of the mass to form an equatorial plate. There are no central bodies visible, but spindle fibres are present. Because of the compactness of this equatorial plate, individual chromosomes are not readily distinguished at this stage. The plate soon splits and the chromosomes start a typical migration towards the poles of the cell. Only after this migration is nearly finished can individual chromosomes be observed. In this late anaphase twelve chromosomes have been counted in each end of a number of cells. The chromosomes are very small and are difficult to count, but there seem to be twelve at each pole at the late anaphase of the primary spermatocytes. Figure 9 is a diagrammatic sketch of the general arrangement of the chromosomes at this stage. They are not as clearly distinguishable as indicated in this figure.

The secondary spermatocytes are readily distinguished from the primary ones by their smaller size. At first the chromatin is in the form of a coarse densely packed mesh. It soon forms a compact knot which gradually changes to the equatorial plate of the spindle. The meiotic process continues without a pause, forming two daughter spermatids. The details of this division are difficult to observe because of the small size of the cells and the masking, caused by the compactness of the chromatin. The chromatin masses can be resolved into individual chromosomes only with great difficulty in the secondary spermatocytes. Figure 10 shows a secondary spermatocyte.

EXPLANATION OF PLATE

The figures of this plate, unless otherwise indicated, represent a magnification of 3500 diameters as they are reproduced. Figure 10 is drawn at a magnification of 4000 diameters. Figure 16 represents a magnification of 700 diameters. The figures were drawn with the aid of a 1.5 oil immersion (Spencer) and a 10 × ocular (Spencer). The drawings are all from sections.

FIG. 1. Resting spermatogonium showing large chromatin-nucleolus.

FIG. 2. Spermatogonium with double nucleolus.

FIG. 3. Primary spermatocyte showing fragmentation of the nucleolus.

FIG. 4. Primary spermatocyte with chromatin in finely granular state.

FIG. 5. Later stage of development.

FIG. 6. Pachytene stage.

FIG. 7. Metaphase of primary spermatocyte.

FIG. 8. Polar view of primary spermatocyte showing equatorial plate in which individual chromosomes are not readily distinguished.

FIG. 9. Diagrammatic sketch of late anaphase showing general arrangement of chromosomes. They are not as distinct as represented in this figure.

FIG. 10. Secondary spermatocyte with massed chromatin.

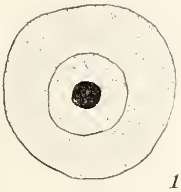
FIG. 11. Spermatid which has begun to elongate.

FIG. 12. Sperm stained with iron hæmatoxylin.

FIG. 13. Sperm stained by Flemming's triple method.

FIGS. 14 and 15. Axial views of the sperm in the region of the knobs.

FIG. 16. Section through the wall of the gonad. *a.* entoderm; *b.* mesoglœa; *c.* spermatogonia; *d.* primary spermatocytes; *e.* secondary spermatocytes; *f.* spermatids; *g.* sperm; *h.* axial view of sperm; *i.* ectoderm.



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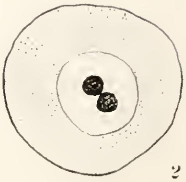

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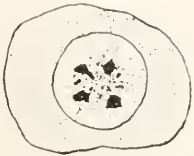

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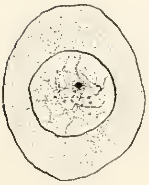

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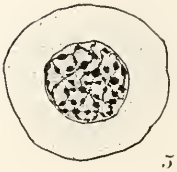

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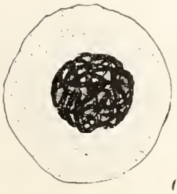

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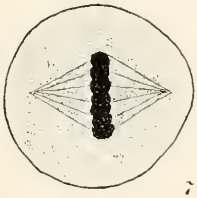

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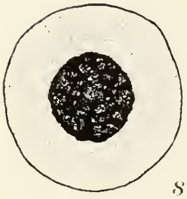

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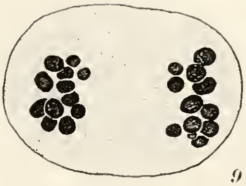

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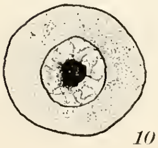

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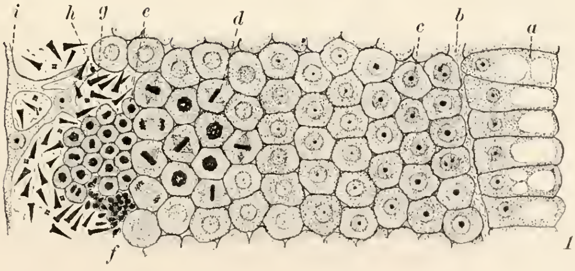

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The spermatids are at first small and round. They gradually draw out at one side. This continues until there is a distinct process projecting from one side, at the base of which are four or five balls or knobs. There are generally four knobs, but five are sometimes present. These knobs stain very intensely with iron hæmatoxylin, but stain only very slightly with safranin. The process takes iron hæmatoxylin stain, but not so intensely as the knobs, and is more readily destained. Figure 12 shows a sperm stained with iron hæmatoxylin. Figures 14 and 15 are axial views of the sperm in the region of the knobs, similarly stained. Figure 13 is a drawing of a sperm stained by Flemming's triple method. There is no indication of a flagellum in fixed material, but Vaney and Conte (1901) observed live sperm and reported the presence of a flagellum at the base of the knobs. If this observation is correct, the long pointed part is the anterior end of the sperm.

Summary

1. Fresh-water medusæ were observed in Stallworth Lake, an artificial body of water near Tuscaloosa, Alabama. They were first seen by the writer on September 14, 1928. They disappeared on October ninth of the same year and have not been observed since.
2. The hydroid stage was not found.
3. A description of the lake where the medusæ were found is given.
4. A historical survey of the fresh-water medusæ is made.
5. The medusæ agree very closely with *Craspedacusta ryderi* as described by Payne (1924). There are certain differences which may be developmental or due to dissimilar environmental conditions. The medusæ are assigned to the species *C. ryderi*.
6. The tentacles are grouped into four, instead of the three previously used groups.
7. Sections of the gonads demonstrated that all the medusæ examined were males.
8. Observations on the spermatogenesis of *C. ryderi* are included.
9. Since all gonads obtained were from adult medusæ, no information concerning the origin of the germ cells can be given.
10. The primary spermatocytes are only very slightly larger than the spermatogonia.
11. There is a meiotic stage in which the chromatin is stained only very poorly with iron hæmatoxylin.
12. There seem to be twelve chromosomes at each pole of the late anaphases of the primary spermatocytes.
13. Some portions of the sperm are stained more intensely by

Flemming's triple method and others by iron hæmatoxylin. Thus the two methods give different appearances to the sperm.

14. Tails are not visible on sperm in fixed and stained preparations.

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THE LONGEVITY OF UNFERTILIZED GAMETES

BENJAMIN H. GRAVE AND JOSEPH F. OLIPHANT

(*From the Marine Biological Laboratory, Woods Hole, Mass.*)

INTRODUCTION

Observations which have been reported on the aging process of unfertilized gametes and upon chemical phenomena associated with fertilization make it desirable that these processes be studied in several widely separated species. It is important to know whether the described phenomena are of wide or of limited occurrence.

The conclusion of Goldforb that the eggs of different individuals of the same species are very diverse, and that failure to take this into account may vitiate experimental studies upon these eggs is a matter deserving consideration, especially by those whose work involves the use of eggs and developing embryos.

The suggestion made by F. R. Lillie that the gametes, both eggs and spermatozoa, contain substances which are essential to fertilization deserves critical attention. It is assumed that these particular chemical substances are gradually lost by the gametes to the water, so that, if a spermatozoön does not promptly meet with an egg after being shed, it loses its ability to fertilize an egg or to initiate the fertilization reaction. This theory accounts for the well-known phenomenon that spermatozoa in dilute suspensions lose their power to fertilize more quickly than is the case with concentrated suspensions. This phenomenon, however, may be accounted for on the theory that the more concentrated suspensions contain more of the longer-lived spermatozoa and therefore appear to retain vitality longer than dilute suspensions. Cohn (1918) explains it by saying that spermatozoa are endowed with a limited amount of energy. When this is used up their movements cease. In concentrated suspensions, because of increased hydrogen ion concentration brought about by the production of carbon dioxide, the activity of the spermatozoa is lessened, and they live for a longer time.

Aside from these more fundamental problems, we have had in mind to determine how long eggs may retain vitality and be capable of fertilization and normal development in case they fail to meet a spermatozoön. In a former paper the senior author published results of experiments upon the eggs and sperm of the lamellibranch, *Cumingia* *tellinoides*, in which it was shown that spermatozoa may, even in dilute suspensions, retain their ability to fertilize eggs for several hours and that so long as they are able to swim they retain this ability.[1] Experiments on the gametes of the annelid, *Hydroides hexagonis*, made during the summer seasons of 1928 and 1929, confirm the interpretation given in the paper on *Cumingia*.

The Egg of Hydroides

The eggs of *Hydroides hexagonis* are comparatively small, measuring 0.057 to 0.063 mm. They are protected by a heavy vitelline membrane, and the germinal vesicle is normally intact until the spermatozoön enters the egg.

They are readily obtained during the summer at any time between June 20 and September 20. However, throughout the early part of the breeding season, the mature are mixed with the immature eggs to such an extent as to render their study difficult. Late in July and during the first half of August the eggs of this species are in their best condition and almost free from immature eggs. Our studies were therefore made chiefly during the height of the breeding period, thus avoiding also the latter end of the season, when the eggs are found to be inferior in vitality and erratic in their behavior. It has become evident that the last eggs produced are in a weakened condition, presumably from too long storage in the cœlomic cavities. Goldforb believes that long storage is the chief cause of the variation in longevity shown by the eggs of *Arbacia*. We believe that this is the cause of the inferiority of the eggs of *Hydroides* which was noticed toward the end of the breeding season.

Longevity of Eggs

In each of our experiments the eggs of eight to ten females, spawned during the same hour, were kept without fertilization in as many Stender dishes. The water was changed frequently to provide aëration and reduce infection. At regular intervals a few eggs from each lot were examined microscopically and note was made of physical changes which indicate deterioration or death by disintegration. At the same intervals a few eggs of each lot were inseminated with fresh sperm to ascertain the rate of deterioration as indicated by the percentage that failed to fertilize and cleave. Numerous experiments of this type were carried out, the results of which show that the poorest lots of eggs disintegrate within six or eight hours, while the longest-lived eggs survive for thirty-two hours. The variation in longevity within a single lot of eggs is greater than in the cases of eggs of other species studied to date. The average longevity of the unfertilized eggs of this species appears to be between eighteen and twenty-four hours. It was shown that the most vigorous lots of eggs that were fertilized after they were from eighteen to twenty-four hours old, gave rise to abundant normal trochophore larvæ. The oldest eggs that gave rise to normal larvæ were twenty-six hours old. Many eggs even from the first die in cleavage stages, and the percentage which fail to become normal larvæ increases with age. Table I shows the variation observed in the longevity of unfertilized eggs. It includes selected examples to show the whole range of variation which has been found by two years study of this species.

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[1] "Vitality of the Gametes of *Cumingia tellinoides*," *Biol. Bull.* 1928.

The shortest-lived lots are given at the top of the table, and it is shown that all of the eggs of females I and II died in eight or nine hours, while a small percentage of the longest-lived eggs of females X and XII were capable of fertilization after thirty-two hours. The average longevity appears to be about eighteen to twenty-four hours.

The longevity of the eggs was tested also by the method of disintegration, a living egg being readily distinguished from one that is dead. When an egg dies, the vitelline membrane remains intact, but the protoplasmic contents either become compact and lobulated like a raspberry or a golf ball, or it disintegrates and the egg becomes swollen.

TABLE I

The comparative longevity of the unfertilized eggs of various females of hydroides hexagonis as indicated by the decreasing percentage of cleavage with age. A few eggs of each lot were fertilized at intervals as shown in the several columns of the table.

Female No.	Percentage of Cleavage with Aging			
	after 1 hour	after 5 hours	after 8 hours	after 10 hours
	per cent	*per cent*	*per cent*	*per cent*
I	80	27	4	0
II	78	60	2	0
III	77	0	65	0
IV	98	0	90	0
V	90	0	90	0
VI	98	0	92	0
VII	92	0	0	0
VIII	99	90	0	0
IX	96	91	0	0
X	92	78	67	0
XI	95	91	0	0
XII	93	0	0	0
XIII	97	96	0	0

TABLE I—*Continued*

Female No.	after 18 hours	after 24 hours	after 28 hours	after 32 hours
	per cent	*per cent*	*per cent*	*per cent*
I	0	0	0	0
II	0	0	0	0
III	3	0	0	0
IV	49	26	0	0
V	26	12	0	0
VI	38	10	0	0
VII	40	16	0	0
VIII	52	6	0	0
IX	22	14	2	0
X	0	48	17	2
XI	0	28	6	0
XII	50	45	12	5
XIII	0	8	0	0

By making counts of the eggs at intervals and applying this test, the variation in the longevity of the eggs of various females was determined.

Table II gives the longevity of the eggs of seven females as tested by the method of disintegration. It shows that the average longevity lies between eighteen and twenty-five hours.

TABLE II

The Longevity of Unfertilized Eggs of Hydroides hexagonis as Tested by the Method of Disintegration

Female No.	Percentage of Disintegration with Aging				
	after 1 hour	after 18 hours	after 25 hours	after 30 hours	after 32 hours
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
I	0	65	93	100	0
II	8	84	90	100	0
III	2	25	40	75	90
IV	0	30	48	85	95
V	2	75	88	100	0
VI	10	100	—	—	—
VII	20	100	—	—	—

THE LONGEVITY OF SPERMATOZOA

The longevity of the spermatozoa of *Hydroides* was studied in dilutions of 1/1000, 1/50,000, 1/100,000, 1/200,000, 1/400,000, 1/800,000, and 1/1,000,000. One drop of dry sperm added to 50 cc. of sea water was taken as a standard sperm suspension of 1/1000. The weaker sperm suspensions were made from this standard suspension by appropriate dilutions.[2]

The questions sought to be answered were three: First. How long will spermatozoa live in sea water under natural conditions and retain their fertilizing potency? Second. Does the spermatozoön contain a soluble chemical substance essential to fertilization, and is this substance lost promptly by the spermatozoön to the sea water, thus rendering it incapable of fertilizing an egg? Third. How great must be the dilution of spermatozoa before capability to fertilize one hundred per cent of the eggs that are added to it is lost? (Is one sperm enough to fertilize an egg?)

TABLE III

Longevity of Spermatozoa of Hydroides, Aug. 16, 1929

Percentage Suspension	Percentage of Cleavage Obtained from Aging Sperm of Various Dilutions			
	after 1 hour	after 3 hours	after 5¼ hours	after 8 hours
	per cent	*per cent*	*per cent*	
1/1,000 or 0.1 per cent	98	98	90	..
1/50,000 or 0.002 per cent	98	98	18	..
1/100,000 or 0.001 per cent	98	85	11	..
1/200,000 or 0.0005 per cent	98	83	17	..

Although a complete answer cannot be given to any of these questions, the data assembled throw light upon all. Tables III and IV show that all sperm dilutions from 1/1000 to 1/1,000,000 give practically 100 per cent fertilization shortly after the dilutions are made and that little deterioration has occurred after three hours. However, it is apparent after three hours that some of the spermatozoa are dying. After five hours the 1/1000 suspension still gives approximately ninety per cent cleavage, while cleavage in the weaker suspensions has fallen to twenty per cent or less. Other experiments show that not more than five to eight per cent cleavage may be expected from suspensions eight hours old. (Table IV) Nearly all of the spermatozoa are dead in dilute suspensions after seven or eight hours. Eight hours may, therefore, be taken as the approximate limit of the life of the spermatozoa of *Hydroides* in dilutions that may be comparable to conditions found in nature.

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[2] The spermatozoa of *Hydroides* may be obtained dry by placing the worm upon a dry glass plate as soon as it has been removed from its calcareous tube. The sperm is promptly expelled from the nephridiopores all along the sides of the body. The pipette used in making dilutions gave twenty drops per cc. One drop of dry sperm in 50 cc. of water therefore gives a 1/1000 or 0.1 per cent. suspension. Ten drops of this standard sperm suspension in 50 cc. of water gives a 1/100,000 or 0.001 per cent sperm suspension, etc. One drop of the standard sperm suspension in 50 cc. of water gives a 1/1,000,000 or 0.0001 per cent sperm suspension.

If the spermatozoa of *Hydroides* contain a substance essential to fertilization, it is lost to the sea water only very slowly, because the spermatozoön retains its ability to fertilize an egg from three to eight hours and probably as long as it is able to swim.

It will be noted from Table IV that suspensions of 1/1,000,000 or 0.0001 per cent are able for a short time to fertilize almost 100 per cent of the eggs that are added to it. The potency of this extreme dilution is a partial answer to Glaser's question: "Is one spermatozoön capable of fertilizing an egg?"

TABLE IV

Longevity of spermatozoa of hydroides, Aug. 18, 1929. This table gives the longevity of spermatozoa from one male in various dilutions, including the extreme dilution of 1/1,000,000 or 0.0001 per cent.

Percentage Suspension	Percentage of Cleavage Obtained from Aging Sperm of Various Dilutions		
	after 1 hour	after 5 hours	after 8 hours
	per cent	*per cent*	*per cent*
1/1,000 or 0.1 per cent	94	85	5
1/50,000 or 0.002 per cent	93	84	1
1/100,000 or 0.001 per cent	93	83	4
1/200,000 or 0.0005 per cent	92	28	2
1/400,000 or 0.00025 per cent	93	26	1
1/800,000 or 0.000125 per cent	91	15	0
1/1,000,000 or 0.0001 per cent	92	8	1

The spermatozoa of *Hydroides* are unusual in one respect. When first expelled into sea water they are inactive and remain relatively so for almost an hour. Due probably to the stimulus of sea water they gradually become active and in the course of about half an hour are vigorous in their movements, but never so active as the spermatozoa of *Cumingia*, *Arbacia* or other species studied. It was anticipated that, because of its comparative lack of vigorous movement, this spermatozoön might survive for a longer period than other sperm, but this does not appear to be the case.

SUMMARY AND CONCLUSIONS

By way of summary, it may be said that unfertilized eggs of *Hydroides* which fail to meet a spermatozoön live and retain ability to be fertilized and develop into normal trochophores from eighteen to twenty-four hours. The extreme variation in the longevity of *Hydroides* eggs is six to thirty-two hours. Some lots of eggs at spawning are in poor physiological condition and one hundred per cent of these die within eight hours. The best lots of eggs, on the other hand, show little deterioration under fifteen hours and a large percentage (40 to 50 per cent) of these are capable of normal development after twenty-four hours.

Spermatozoa, when expelled into sea water or mixed in dilute suspensions comparable to that obtaining in nature, live from three to seven hours and retain ability during that time to fertilize eggs.

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THE GOLGI APPARATUS OF AMOEBA PROTEUS PALLAS

V. E. BROWN

University of Maryland, School of Pharmacy

Introduction

The study of the Golgi apparatus and its identification in the Protozoa has been rendered almost impossible because the highly specialized Golgi techniques are not very specific, and they are very capricious even in the hands of our best technicians. Bowen (1928) has pointed out the fact that isolated gland cells often fail to respond to impregnation methods, and he suggests that similar trouble may be expected in protozoan techniques. Also the identification of the Golgi apparatus is impeded because just what may or may not be Golg. material has not been agreed upon by many of the investigatorsi Hirschler (1914) found spheres, crescents, and rings in *Monocystis agilis* and *Gregarina polymorpha*. King and Gatenby (1923) described similar crescents, and bead-like structures in *Adelia*. They believe these structures to be comparable to the dictyosomes of metazoan cells. Nassonov (1924, 1925) believes that the contractile vacuole is the homologue of the Golgi apparatus of metazoan cells. He found that the contractile vacuoles of *Paramecium*, *Lionotus*, *Chilodon*, and *Dogielella* stained by the Kolachev method of procedure. Duboscq and Grassé (1924–1927) believe that in *Holomastigotes*, *Pyrsonympha* and other related flagellates the parabasal bodies are the homologues of the Golgi apparatus. Grassé (1926) stated that since the euglenoid flagellates have no parabasal bodies, the stigma or eye-spot is a homologue of the Golgi apparatus. Causey (1925) finds a network in *Endamœba gingivalis;* this structure is similar to the Golgi network in metazoan tissues. Hirschler (1927) found rings, spheres, and crescents in *Endamœba blattæ* and he believes that these are the Golgi material. Joyet-Lavergne (1926) used neutral red as a vital stain on gregarines and found that these crescents and rings stained; therefore, he believes the structures to be Golgi elements. Hall (1930) finds in *Chilomonas paramecium* small granules and large vacuoles, and he believes that these bodies are comparable to the Golgi apparatus.

There is a great divergence of opinion as to just what may be or may not be Golgi material in the Protozoa. The structure and function of the Golgi apparatus are subjects of controversy. It is the aim of the writer to describe and discuss the Golgi apparatus of *Amœba proteus* Pallas.

This work was done in the Zoölogical Laboratory of Johns Hopkins University and the writer wishes to take this occasion to thank Dr. Samuel O. Mast for numerous kindly favors and helpful coöperation.

Material and Technique

Amœba proteus was cultured on a cracked wheat infusion which was inoculated with *Chilomonas paramecium* and *Zoöchlorella*.

For fixation the centrifuge method of procedure was used. The Kolachev method as modified by Nassonov (1925) and Bowen's modification of the Mann-Kopsch procedure were used to good advantage. The Mann-Kopsch method gave the best results. Also Bowen's acid fuchsin-thionin-aurantia was slightly modified and used after a fixation in Champy's solution.

(1) Fix in Champy's solution 12 hours.
(2) Wash in distilled water.
(3) Pass the slides through graded alcohols to 70 per cent.
(4) Stain 10 minutes in acid fuchsin.
(5) Stain 15 seconds in very dilute thionin.
(6) Stain 5 seconds in dilute aurantia.

Dehydrate and mount in balsam.

Result: Golgi bodies are light blue with dark blue rims, the cytoplasm is pink, the nucleus is orange or vermillion, and the contractile vacuole and the granules around it are red.

The Golgi Apparatus of Amoeba

The Mann-Kopsch method of procedure brings out two types of granules in *Amœba proteus* which reduce osmic acid. One of these is a black granule and the other is a spherule with a black rim (Fig. 2, Plate). The black granules are similar to those described by Hall (1930) as occurring in *Chilomonas paramecium*. When the spherules are viewed at a central focus, they appear as rings, or crescents (Figs. 1–2, Plate). These bodies have clear centers. Both types are distributed at random throughout the endoplasm of *Amœba*, and often they have a tendency to flow out into the pseudopodia (Fig. 2, Plate). In no cases have they been observed to be associated with the contractile vacuole. There are small granules which group around the contractile vacuole. These do not take an osmic stain, but they are easily demonstrated by acid fuchsin (Fig. I, Plate).

The contractile vacuole of *Amœba proteus* does not take an osmic stain; however, *Paramecium caudatum* occurs in the same slides and its contractile vacuoles are blackened by osmic acid as described by Nassonov (1925). Small vacuoles of variable sizes have been observed to be attached to the spheres with dark rims (Figs. 1–2, Plate). These are also noticed in the endoplasm; they are numerous around the contractile vacuole (Figs. 1–2, Plate). This causes the writer to believe that these small vacuoles originate from small granules which move about in the endoplasm and group around the contractile vacuole. They evidently flow together to form the new vacuole after the systole. The formation of the contractile vacuole is different therefore, from *Paramecium caudatum*, where the contractile vacuoles with their feeding canals are more or less permanent structures. The black granules are of variable sizes, and it is possible that these grow in size to form the spheres with black rims. The reaction of the acid fuchsin-thionin-aurantia and osmic acid procedures indicate that both types of bodies are Golgi material (Figs. 1–2, Plate). Also these bodies are similar to those described by Hirschler (1914), King and Gatenby (1923), Joyet-Lavergne (1926) and Hall (1930).

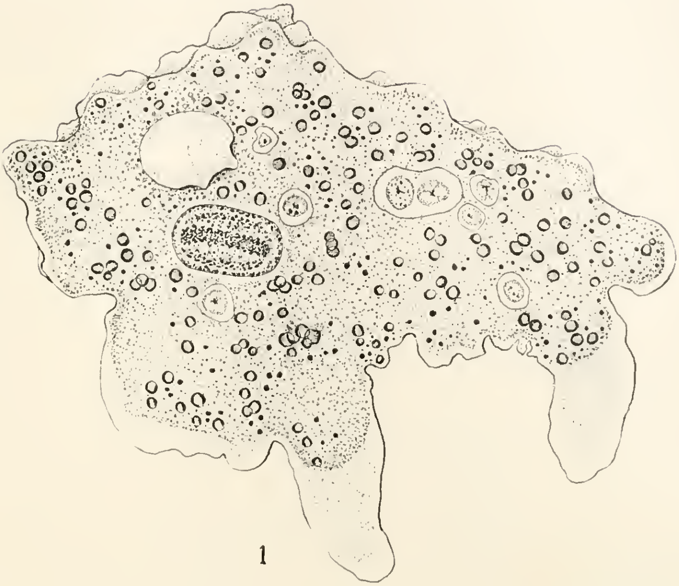
Bowen's modification of the acid fuchsin-thionin-aurantia method of procedure stains the Golgi apparatus of *Amœba proteus* blue. The spheres have dark blue rims (Fig. 1, Plate), whereas their centers are light blue. The Mann-Kopsch procedure stains them black; the large ones have light centers with black rims (Fig. 2, Plate).

Discussion

Hirschler (1914) described the Golgi apparatus (*i.e.*, spherules) of *Monocystis ascidæ* as rings and crescents. These have dark rims with centers. King and Gatenby (1923) described similar bodies as occurring in *Adelia*. They declared that these crescents and bead-like structures were comparable to the dictyosomes of metazoan cells. They also stated that these dictyosomes divide like those of the metazoan cells.

Hirschler (1914) found that the Golgi material of the Protozoa was made up of two substances, one of which takes a light stain, the other a dark one. Such a staining reaction occurs in the Golgi apparatus of *Amœba proteus*. One portion is chromophilic and the other chromophobic. This probably accounts for the fact that the spherules have dark rims. However, the writer does not find that the chromophobic portion has an affinity for acid fuchsin.

It is probably of interest to remark here that Vonwiller (1913) finds that neutral red used as a vital stain on *Amœba* colors the crystal-vacuoles (vacuoles which contain the characteristic crystals of *Amœba*)



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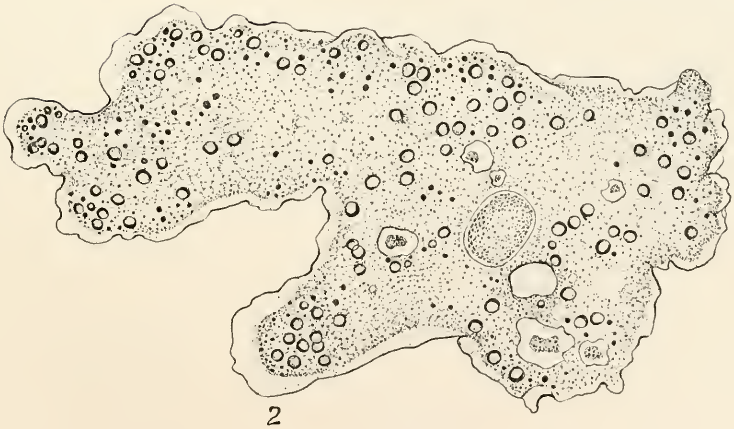

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Explanation of Plate

Both figures were drawn with the aid of an Abbé camera lucida. The chondriosomes were omitted in the drawings in order to avoid confusion.

FIG. 1. *Amœba proteus* fixed in Champy's solution and stained with acid fuchsin-thionin-aurantia. The Golgi bodies occur in clumps. The large globules have clear centers and dark rims. This gives the appearance of rings and crescent-shaped structures. The small globules are dark blue.

FIG. 2. *Amœba proteus* fixed and stained by Bowen's modification of Mann-Kopsch procedure. The Golgi bodies have a tendency to flow out into pseudopodia.

orange, whereas the smaller granules stain red. Joyet-Lavergne (1926) finds that vital staining with neutral red brings out the Golgi apparatus in the gregarines. These bodies occur as rings, crescents and spherules with dark red rims. Hall (1929) finds that neutral red used as a vital stain demonstrates the Golgi apparatus in various Protozoa. Hall (1930) finds similar globular bodies in *Trichamœba*, which react to neutral red and osmic acid. Occasionally crescent-shaped structures are noticed. Hall (1930*b*) shows that these globules are stained selectively with neutral red and are the same bodies which are blackened by osmic acid and silver impregnation. He used a mixture of Janus green and neutral red to distinguish these globules from the mitochondria of *Trichamœba*. The writer finds similar globules and crescent-shaped structures in *Amœba proteus* (Figs. 1–2, Plate). These bodies are smaller than the "crystal vacuoles," as described by Vonwiller (1913), but they are probably the same globules which stain bright red. These bodies have a tendency to flow out into the pseudopodia as described by Vonwiller. Therefore these globular bodies and crescent-shaped structures are probably the Golgi apparatus of *Amœba proteus* (Figs. 1–2, Plate).

Bowen (1923–1929) has suggested that the Golgi apparatus has a secretory function. He finds that the Golgi apparatus of glandular tissue hypertrophies at the beginning of the secretory cycle; when it hypertrophies, spherical and globular bodies are formed. These globular bodies have clear centers and dark rims when impregnated with osmic acid and are similar in many respects to those found in the Protozoa. A true Golgi network is not found in *Amœba proteus*, and these crescents and the globules are similar to the globules described by Bowen (1926). The writer is led to believe that the Protozoa are cells where secretion is a constant process; therefore, the Golgi apparatus would naturally be expected to be hypertrophied and occur as globules.

It has been suggested by Bowen (1926) that the relation between the Golgi apparatus and the secretory granules is homologous to that existing between the Golgi apparatus and the developing acrosome of vertebrate sperm, and this latter phenomenon can be used as a basis for interpretating the phenomena in the gland cell. The writer believes that a similar relationship exists between the crescent-shaped Golgi bodies and the minute vacuoles which occur throughout the endoplasm of *Amœba proteus*. These minute vacuoles are often attached to these crescent-shaped bodies (Fig. 2, Plate). They also occur throughout the endoplasm, and similar vacuoles occur in large numbers around the contractile vacuole. These vacuoles break into the contractile vacuole when the systole occurs. Day (1927) finds that the contractile vacuole of *Amœba proteus* arises from the fusion of small vacuoles which probably owe their origin to the fusion and coalescence of ultra-microscopic droplets of soluble katabolic waste which may include the water of osmosis. The minute vacuoles described above seem to be similar in all respects to those described by Day (1927). The vacuole of *Amœba* is not a permanent structure and is formed by the fusion of minute vacuoles; this differs from the contractile vacuoles of *Paramecium caudatum,* where two permanent contractile vacuoles occur and pulsate successively. The contractile vacuoles of *Paramecium caudatum* have long "feeding" canals, and these structures are blackened by osmic acid (Nassonov, 1925).

Paramecium occurs in my slides along with *Amœba proteus,* and the contractile vacuoles of *Paramecium* are blackened by osmic acid; whereas, the contractile vacuole of *Amœba proteus* is not stained. This may be due to the difference in the formation of these vacuoles in the two Protozoa.

General Summary

1. The Golgi apparatus of *Amœba proteus* is the characteristic protozoan type of globules and spherules with clear centers and dark rims. These spherules, from a central focus, appear to be crescent-shaped structures. Small black granules appear without black centers.
2. These Golgi bodies are readily blackened by osmic acid and stained with thionin.
3. At the beginning of the secretory cycle of metazoan gland cells, the Golgi apparatus hypertrophies and globules are formed; the globules of *Amœba proteus* are believed to be homologous to these structures.
4. Since the secretory cycle is a continuous process in *Amœba,* it is suggested that this is the reason for the absence of a Golgi network.
5. It is suggested that the minute vacuoles which occur in the endoplasm of *Amœba* are associated with the crescent-like Golgi bodies, a relationship which is similar to that existing between the metazoan Golgi apparatus and the secretory cycle.
6. The contractile vacuole of *Amœba* is formed by a union of these minute vacuoles. It is possible that this is the reason why the contractile vacuole of *Amœba* is not blackened by osmic acid like the contractile vacuoles of *Paramecium caudatum.*

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THE CHROMATOPHORES OF THE CEPHALOPODS[1]

ENRICO SERENI

Laboratorio di Fisiologia della Stazione Zoologica di Napoli

Ladies and Gentlemen: It is with the greatest pleasure that I am speaking this evening, as a member of the Stazione Zoologica of Naples, here in the auditorium of the Woods Hole Laboratory. I am glad to bring to every one of you the best greetings of us all, and to assure you that, when three years hence you come to Italy to the next Physiological Congress, and next year when some of you go to Padua for the Zoölogical Congress, we hope you will gather in the Stazione Zoologica in as great a number as you have gathered here. I do not know if we shall be able to arrange things as well as you did here, but I can promise that our welcome will be as hearty as yours and that we shall do our best to this purpose.

Ladies and gentlemen, it is impossible to look at the beautiful play of the chromatophores of the cephalopods—the object of our lecture this evening—without being filled with wonder at the complication and the perfection of the play itself. Old Aristotle was the first to study these animals with the eyes of the scientist, and he was also the first to connect some of the different appearances shown by the animals with various conditions of their minds: that is, fear and anger, and so on. He was also the first to suggest that these animals were able to change their colour according to the colour of the environment, giving, in this way, one of the first instances of this fundamental law of living beings, namely, mimicry. It may be of interest to remember that this opinion of his has been confirmed in a most brilliant way quite recently by the careful experiments of some German scientists (Kuehn and Heberdey, 1929).

As in many other instances, Aristotle was more than twenty centuries ahead of his time. It was not until the beginning of the nineteenth century that people began to study again the characters and the mechanism of the colour-changes in cephalopods. Opinions on the nature of colour-changes differed very widely at that time, and some of them may be quoted as instances of the ways in which, if not controlled by observation and experiment, scientific thought can be misled. It was believed at first that the colour was produced by some coloured liquid; but very soon it was discovered that the coloured spots were stable and that they did not change their place, but only their form. They were constant formations, differentiated from some part of the skin. The name of Sangiovanni should be remembered at this point, because he was the first to recognize (1819) that these colour-changes were the effect of some special little organs. To these he gave the name *chromophore*, which has ever since clung to them, although in a slightly modified form, *chromatophores*, that is to say, colour-bearers.

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[1] Lecture delivered at the Marine Biological Laboratory, Woods Hole, Massachusetts, September 13, 1929.

After this first fundamental discovery, which laid the foundation of all subsequent morphological investigations, research-work developed along different lines. It would take us too long to follow closely their development through the nineteenth century to the present time. Out of this work two points have become clear, which it would be well to emphasize now in order to get a better understanding of what we are going to say later on. The first is that the expanded condition of the chromatophores is the active one, just the opposite of what we are accustomed to find in the vertebrates, for example, in the frog. This fact may give rise to several misunderstandings and misinterpretations, because of the opposite meaning that the same word is bound to have when applied to the chromatophores or to the muscular fibers. In order to avoid these misunderstandings, we shall, once for all, decide, after Steinach, to speak of expansion and retraction of the chromatophores, to which correspond respectively the contraction and relaxation of the muscular fibers. The second point of importance was the definite recognition (by Steinach, 1901) of the muscular nature of the radial fibers of the chromatophores, a fact which was established after manifold and lengthy debates, in spite of the strenuous opposition of some investigators. These two facts are undoubtedly connected, and they both bring us to consider more closely the morphology of the chromatophores.

In the frog, as well as in other vertebrates and in the crustaceans, chromatophores are single cells, whose contractile properties resemble more nearly amœboid contractility than that of even slowly contracting muscular tissue. In the contracted (or "active") condition, the chromatophore looks like a point or like a little sphere and resembles an encysted amœba. From this extreme contracted or active condition all stages up to extreme expansion can be observed. Little by little the chromatophore cell gives out prolongations—we may as well call them pseudopodia—which expand more and more. At the end they are so widely expanded as to penetrate between the neighbouring chromatophores and to form in this way an almost continuous layer of coloured cells.

The chromatophores of the frog, and of vertebrates in general, are therefore to be considered as a very peculiar sort of formation; together with the leucocytes, the only representatives, in the realm of more highly organized beings, of cells endowed with this fundamental property of protoplasm, *i.e.*, contractility, in its most elementary form.

The case of the chromatophores of the cephalopods is completely different. It cannot be our task to discuss the question of their descent from one or more cells. What matters for us is that in their definitive condition they are formed by a sort of little sack and by a series of radially distributed fibers. The sack is filled with pigment granules and its walls possess elastic properties, which are the primary cause for the rounded, or at least elliptical, shape of the chromatophores in the resting condition. The fibers, on the other hand, have their movable end on the walls of the sack, their fixed one somewhere in the skin. The movable ends on the wall are at regular intervals, arranged in such a way that the whole looks like a radial figure. The fibers, as mentioned above, are muscular in nature, and therefore, every time they are reached by a stimulation and contract, the walls of the sack become expanded and remain expanded as long as the contraction of the fibers lasts. As soon as the fibers relax, the wall of the sack, because of its elastic properties, goes back to its resting, *i.e.*, retracted condition.

In some instances it can be noticed that under certain natural or experimental conditions the chromatophores may retract still more. To this day it cannot be decided whether this depends upon the inhibition, in these cases, of a sort of residual tonus of the fibers, which generally hinders the full action of the elasticity of the wall, or upon the existence of some sort of device for the purpose of active retraction. This last has been postulated by some authors, but, although it cannot be definitely denied, no satisfactory evidence of anything likely to produce such an effect has been reported so far.

It should be clear from this description that the expansion of the chromatophores is produced by the combined pull of many (sometimes 20–25) fibers at the same time. Every one of these individual fibers pulls in its own direction, but, as they are radially distributed, it follows that the chromatophore expands as a whole and almost maintains its form, which may now be described as a more or less regular polygon, whose rather rounded edges correspond to the points where each fiber is attached.

As mentioned above, under "normal" conditions all fibers of a given chromatophore pull at the same time, and as they have no direct connection whatever between themselves (excepting through the wall of the chromatophore itself), this fact, which is one of very perfect coördination, points to some kind of external control. As a matter of fact, under normal conditions the chromatophores are strictly and continuously controlled by the central nervous system. As soon as the connection with this is severed, the chromatophores are completely paralyzed and, for some time at least, they remain in a condition of complete rest unless artificially stimulated by way of the peripheral nerves. The demonstration of this fact, for which we are indebted to Leon Fredericq (1878), is a very easy one. If, in any species of cephalopods, the so-called mantle-connective is cut, the chromatophores of the corresponding half of the body, as well as the mantle-muscle itself, are instantly and forever paralyzed. However excited the animal may be, however beautiful and intense the play of its chromatophores on all other parts of the skin, those on the half whose nerve has been cut will never again reproduce the same phenomena. The play of the chromatophores, the normal one at least, is produced by impulses coming from the central nervous system: the mantle-ganglion, which, in the experiment just described, is still connected with the chromatophores, is completely unable to produce any spontaneous or reflex expansion. It seems that the ganglion has nothing to do with the chromatophores of the mantle, and that the nerves to these last go directly, without any stop, from the central ganglia to the peripheral plexi.

Now we begin to know something about the forces controlling the chromatophores. The next problem to consider is that of the afferent paths of the reflex arc whose efferent paths are represented by the nerves going to the chromatophores: that means that it is necessary to decide to which stimuli the centers for the chromatophores respond.

We have related above the answer which Aristotle gave to this question. He admitted, rather briefly we must say, that anger and fear and similar feelings were the primary cause for the colour-changes of the sea-devils and of the squids. We will not deny this opinion; much more, we shall support it in a rather unexpected way. A physiologist, however, cannot remain satisfied with these rather vague expressions and is bound to analyze these phenomena somewhat more deeply. The analysis has been carried on by different authors and the importance of several sorts of stimuli has been recognized.

First of all, it is through the eyes that the animal receives the impressions that produce the changes in its colour. Anybody may notice that the colour of the animal changes whenever it sees any object which attracts its attention. That is the phenomenon noticed by Aristotle. It is possible, however, to have some more exact idea of the importance of the visual impressions for the colour-changes by determining how far the animal still maintains its capacity for changing its colour when the function of one, or both eyes is excluded. The play of the chromatophores is not abolished in either case, but the capacity which the normal animal has for changing its colour with the colour of the environment is lost. For this sort of adaptation at least, visual impressions are of the first importance; but, in contrast to what happens in some fishes, they are not the only ones controlling the expansion of the chromatophores. It is interesting to remember that when only one eye is put out of function, the activities of the chromatophores of the corresponding half are somewhat diminished. They answer to all stimuli as well as those of the other half, but somewhat more slowly, and their expansion is often less complete. This phenomenon shows that although the stimuli reaching each eye act on all coloration centers of the central nervous system, they affect primarily those of the corresponding half, and their action on those of the opposite half is somewhat more indirect.

The animal deprived of its visual impressions still maintains its capacity for changing its colour, although this function is somewhat impaired. It was the merit of Steinach (1901) to demonstrate that another set of impressions controlling the play of the chromatophores is that issuing from the suckers. There is no doubt that the importance of these excitations for the play of the chromatophores was somewhat exaggerated at first, but it is impossible to deny that, if all suckers are extirpated, the "tonus" of the chromatophores is much diminished, and their movements much less frequent and vigorous. The animal, deprived of its suckers, or of both suckers and eyes, has not yet lost its colour-controlling capacity, although this is much impaired. It still answers with a change of colour, just as does the normal animal, to many kinds of stimuli: for instance, to vigorous stirring of the water (which probably amounts to both an excitation of the sense terminations in the skin and of the statocysts), to pricking or pinching the skin, placing on its back, and so on.

The phenomena we have just described show that there are not specific stimuli affecting the centers of the chromatophores. The latter may answer a variety of excitations, none of which can be described as alone responsible for the changes of colour, although any one of them can, at any given moment, induce them. The center, or better, as we shall see later, the centers for coloration are the effector centers for many, we may as well say for all, sorts of excitations, every one of which may affect them in turn, or together with others. The excitations from the eyes and from the suckers assume a somewhat more prominent place. This is only because, more often than the others, they affect not only the momentary conditions of the chromatophores, but also their "tonus," that is to say, the condition of greater or lesser expansion which prevails for some time and on which the momentary changes superpose.

There is a third group of excitations of which little or no notice has been taken until now, and which, without any doubt, along with those from the eyes and suckers, plays a very important part in determining the "tonus" of the chromatophores in the normal animal (Sereni, 1928*a*). It is easy to notice that the ventral side, both of the body and the tentacles, is generally less coloured than the dorsal one. This depends partly upon a morphological difference, namely, a somewhat different distribution of the chromatophores. A closer observation, however, shows that the chromatophores of the ventral side are much less expanded. This fact corresponds to a very general phenomenon among sea animals, whose ventral side is very often less coloured than the dorsal one. This may be considered as a sort of protective coloration because, when seen from below, the sea surface has a whitish colour. However this may be, there is a more immediate cause for it. In the cephalopods the difference in the grade of expansion between the chromatophores of the ventral side and those of the dorsal side is specially marked when the animal lies on the ground or is fixed to the walls of the basin. In such cases, as anybody can see, there is no danger of the animal being attacked from below and any sort of protective device is excluded. It seems much more likely that in this case we have to do with a sort of postural reflex of the chromatophores. As a matter of fact, if, in the normal animal by some special trick, we succeed in placing the animal on its back or on one of its lateral sides, we find that the chromatophores are less expanded upon whichever side the animal is lying. This is more easily done with animals deprived of their eyes and of their suckers. If we turn the animal on another side, the change in the grade of expansion of the chromatophores is immediate. The mechanisms concerned work, therefore, in a very prompt and perfect way. It might be imagined that the chief action is due to the different illumination of the various sides, the less illuminated being also that in which the chromatophores are less expanded. It is, however, easy to show that powerful illumination of the paler side does not alter the result. Nor is the lesser expansion due to any effect of contact or pressure on the chromatophores themselves. The effect of these factors would then be directly the opposite, namely, an increase in the expansion. The effect goes through the nervous system, it appears, not only on the chromatophores touching the ground or the wall, but on the entire half upon part of which the animal lies. It seems safe to conclude, therefore, that from the part of the body upon which the animal lies impulses are sent to the centers of the chromatophores of the corresponding area of the skin, the effect of which is a diminution of the "tonus" of the centers themselves. This fact adds a new and important link to the series of factors acting on the chromatophores and gives us a new instance of postural reflexes, and one belonging to a new class.

We are now in a position to form an opinion, as clear as present day knowledge allows, about the external factors influencing the chromatophores. A very simple experiment, however, namely, the section of any nerve going to the chromatophores, will show us that when these are separated from the central nervous system, none of the stimuli quoted above will act on them in the described way. We are therefore bound to conclude that all these stimuli influence the chromatophores through a nervous mechanism, which means that in reality they are not acting on the chromatophores themselves, but on their nerve centers. The time has come to say something about the centers themselves. It is not a part of our task this evening to expose at length the anatomical facts regarding the central nervous system of the cephalopods. It need only be remembered that it is the most highly organized and the most centralized among those of the invertebrates, the various ganglia running together to form a sort of brain. The importance of this is such that it has developed a protecting device,—a sort of cartilaginous skull. This "brain" is large enough to allow an experimental analysis of its various parts by means of the classical methods of stimulation and extirpation. In relation to the chromatophores, this analysis has gone so far as to show the existence of several centers for the chromatophores of the different parts of the skin. While these centers are located in different parts of the brain and are of a motor kind, Phisalix (1892, 1894) was able to demonstrate the existence in the highest ganglia, the so-called cerebral ganglia, of an inhibitory center acting on the chromatophores of the same side.

This interesting observation by Phisalix was not confirmed until a few years ago I undertook the task of examining the whole question of the central control of the chromatophores. In the study of this problem I availed myself not only of the classical methods of analysis by stimulation and extirpation, but also of the finer one offered by the use of drugs. I was then, and I am now, very well aware of the dangers connected with this method, and I readily acknowledge that its results are subject to criticism and are to be accepted with more reserve than those obtained by the former methods. At the same time, however, I am of the opinion that the results obtained by means of pharmacological analysis, especially if checked with those obtained by some other method, afford a very valuable help in carrying the analysis to a point where neither the scalpel nor the electrode, however delicate and however skillfully handled, could arrive.

As a result of this study, I am now able to confirm the data of Phisalix and to add something to them (Sereni, 1928*a*). The chromatophores are ruled by three different kinds of centers, all of them situated in the brain. The first kind are the purely motor (*B*) centers. They are located in the different subesophageal ganglia, are distinct for the two sides and for the different areas of the skin, and they cannot substitute for each other. These centers are overruled by a general coloration-center (*A*), probably to be located in the so-called central ganglia, and by an inhibitory center (*C*), corresponding to that of Phisalix, in the cerebral ganglia. Both these centers, the general coloration and the inhibitory one, although symmetrical, may act also on the opposite side, and the center on one side may permanently substitute for the other. Their relations to the purely motor (*B*) centers may be considered as similar to those between the cortical and the medullary centers in the vertebrates.

This description makes it perfectly clear, I hope, that the nervous mechanisms connected with the chromatophores are very complex indeed. It is interesting to observe that, in this case as in many more, the exactness and the delicacy, as well as the rapidity and the adequacy of the regulation, has been attained in the same way, namely, by the existence of a double innervation, excitatory and inhibitory. The ways and the methods nature employs in its laboratory are not so manifold as we sometimes fancy they are.

These have not been the only results achieved with the pharmacological analysis. When studying the action of the various drugs on the centers just described, I noticed (Sereni, 1928*b*) that they could easily be grouped, according to their action, into two classes. In the first were the substances producing an expansion of the chromatophores; in the second those inducing a retraction. This depends on the action of the different drugs upon the one rather than upon the other center. There is nothing astonishing in it, nor in the fact that the same effect, for instance, the expansion, can be attained either by a stimulation of the excitatory center or by a paralysis of the inhibitory. It is, however, rather unexpected to find that the grouping of the drugs corresponds very exactly to that with which we are well acquainted from the pharmacology of vertebrates, in sympathetico-mymetic and parasympathetico-mymetic drugs. The fact is that the former ones, for instance adrenalin, increase the expansion of the chromatophores, that is, the "tonus" of their centers; the latter ones, for example pilocarpin, decrease it by stimulation of the inhibitory center, which, on the other hand, is paralyzed by atropin.

The significance of this observation is increased by the fact that the similarity in the action of the drugs of each group among themselves does not limit itself to the action on the chromatophores, or, better, on their centers, but extends to many more functions. We may quote, for instance, the function of the ink-bladder, that of the iris, the respiratory action, and the activity of the muscles in general. On all these functions the drugs of each group act in the same way; the two groups, however, in opposite directions. Since the similarity with the action on the chromatophores is as exact as could be desired, we may conclude that all these functions are ruled by a double set of centers, excitatory and inhibitory, on each of which acts one of the two classes of drugs described above.

It can be added that the similarity in the grouping of the drugs according to their action in the vertebrates or in the cephalopods is a very exact one, not only in the broad lines, but also in particulars. All this seems to show that this is not due to chance.

There is, however, a difference, and, apparently, a very deep one, between the vertebrates and cephalopods. It is a well-known feature of the action of the vast majority of the drugs just named that in vertebrates they act at the peripheral end of the nerve; chiefly in some part of the junction between nerve and effector organ. In the cephalopods, on the other hand, all drugs act, at least in regard to the actions we are now considering, on the central nervous system. This seems to be a very deep difference, and a very important one too. On the other hand, I may say that this difference, however important it looks, could be expected beforehand. As a matter of fact, and as we shall see more clearly in a later stage of our examination, the conditions existing between nerve and effector organ in the cephalopods differ very widely from those in the vertebrates. For example, no morphological differentiation such as the motor endplates has been detected so far in the muscles of the cephalopods; nor does curari act on the latter in its usual and so characteristic manner. These observations may help to explain the reason for this one great difference among so many similarities. On the other hand, we must remember that the methods employed simply allow us to tell that the different drugs act on the central nervous system, or, better, on parts of it. With them, however, we are unable to determine the nature of the elements on which the drugs are acting, and it is quite possible that some of them at least do not act on the cells proper, but rather on the synapses, that is, on the nerve endings of the cells connected with those of the center on which the drugs seem to act.

Whatever may be the conclusion on this point, it cannot be denied that the pharmacological affinities in the vertebrates and in the cephalopods are very similar indeed, and this striking similarity cannot be without some deeper meaning. We know that in the vertebrates two at least of the substances acting on the two sections of the autonomic nervous system, namely, adrenalin and choline (or acetylcholine) are to be found in the body itself. These same substances are among those acting (with opposite results) in the cephalopods. The next step was therefore to look for the presence of these same or of some related substances in the cephalopods. Neither adrenalin nor choline has ever been recorded in the cephalopods. There are, however, at least three substances, tyramine and histamine on one hand, betaine on the other, whose action in the cephalopods is very similar to that of adrenalin and of choline respectively. These substances have been shown to exist in the body of the cephalopods, and the two first to circulate in the blood. We have, therefore, a very close correspondence with the facts in the vertebrates. In both cases substances exist in the body of the animal and in its blood which are pharmacologically active on the animal itself. It seemed rather improbable that this occurrence should be only casual, and it was worthwhile to try to find out if the circulating substances have any physiological importance. A first approach to the experimental solution of this problem was made possible by a fortunate occurrence. There are two very closely related genera, *Eledone* and *Octopus* and two species of *Octopus*, *macropus* and *vulgaris*. Of these *Eledone* and *Octopus macropus* are normally much more coloured, *Octopus vulgaris* much paler. In other words, in the first two normally the general coloration-center is dominant; in the third the inhibitory one prevails. If now the blood of *Eledone* or of *Octopus macropus* is injected in the blood stream of *Octopus vulgaris*, the latter becomes for a while much more coloured, and on the other hand, if the blood of *Octopus vulgaris* is injected in *Eledone* or in *Octopus macropus*, they become paler (Sereni, 1929*a*). The same experiment may be performed by connecting the two animals in crossed-circulation preparation which I have devised for this purpose and which has been described elsewhere.

The results of this simple experiment show in a very clear and definite manner that the circulating blood has some property of modifying the conditions of the centers of the chromatophores. That this property in the case of *Eledone* and *Octopus macropus* is connected with the presence of tyramine or histamine, or both, is made probable by the fact that these substances are much more abundant in the blood of *Octopus macropus* and *Eledone* than in that of *Octopus vulgaris*. The probability becomes a higher one by the demonstration that in conditions when, after an experiment of Bottazzi's (1924), tyramine or histamine are known to be secreted in the blood, the expansion of the chromatophores increases. The final proof was given recently (Sereni, 1929*b*), when, by the extirpation of the so-called posterior salivary glands, (which, as the result of the work of Henze and Botazzi, are known as the organs where tyramine and histamine are chiefly produced or accumulated) I was able to induce in *Octopus macropus* and *Eledone moschata* a condition of complete paleness and general atonia. This condition persists and the animal eventually dies, but it is possible to correct this state by the injection of tyramine and histamine or of the blood of a normal animal.

The work just described proves beyond any reasonable doubt that what we had guessed as probable, namely, that the presence in the body of pharmacologically active substances could not be without some physiological significance, is true. It gives at the same time the first definite and, I daresay, conclusive proof of the existence of phenomena of internal secretion in the cephalopods and in the molluscs in general and one of the very first demonstrations of the same phenomena in invertebrates.

Let us now, after this rather long, but necessary, parenthesis, go back to the physiology of chromatophores. From this standpoint the results quoted above give us the final link in the long chain of phenomena connected with the regulation of the chromatophores. The centers in the central nervous system, controlling the chromatophores (as well as those connected with many other functions) do not only respond to external factors like those previously described; they are also under the continuous influence of internal, humoral factors. These produce and maintain the tonic basal condition, on which the relatively quick changes induced by external factors take place. The effect of these different controls is to make the regulation much more delicate.

There is another point I should like to bring to your notice. It has been the merit of Redfield to show, some years ago, that the chromatophores of a lizard are subject to a humoral control. His results have been widely confirmed and extended in regard to the chromatophores of the vertebrates (Hogben, 1924). Now my results proved that similar phenomena occur also in the control of the chromatophores of the cephalopods, and almost at the same time Koller (1928) and Perkins (1928) were able to show that the same holds for the chromatophores of the crustaceans. There is one difference, however, between the working of the humoral control in the vertebrates and in the cephalopods. In the vertebrates it acts on the chromatophores themselves; in the cephalopods, on the other hand, the humoral control works through the nervous system. That means, it influences the centers controlling the chromatophores. The explanation of this difference may most probably be found in the much higher complexity and development of the chromatophores in the cephalopods.

I feel that this lecture has already been long enough, and I will not detain you much further. Our survey of the physiology of the chromatophores would be, however, very incomplete indeed if, after considering them from the standpoint of their regulation, we should not examine them for themselves. In the first place, when in connection with the central nervous system, the chromatophores are more than anything else to be considered as a sort of indicator, and a very valuable and suitable one, of phenomena happening elsewhere, chiefly in the central nervous system itself. It is possible, however, to consider the chromatophores—or better, in this instance, their radial fibers—from the standpoint of muscular physiology. The chief interest of this study lies in the fact that we have to do here with single muscle-fibers such as there are not many opportunities to find elsewhere.

All the experiments which I have described heretofore have been performed on chromatophores connected with the central nervous system through nerves. From now on we shall consider experiments on the chromatophores deprived of this connection. It would be, however, a very misleading procedure to consider the chromatophores of every part of the skin whose nervous connections have been previously severed as deprived of every nervous influence and therefore showing simply the properties of the muscular fibers alone. The skin is so rich in nervous branches, connected to form different plexi, that whenever a stimulus is applied, it is through the nerves that the excitation reaches the muscles. This is the conclusion derived from the character of the responses, which are identical with those produced by the stimulation of the nervous trunks. When, however, enough time is allowed to pass after the section of the nerve, or when the animal has been dead for some time (the actual length of time varying in both instances with the season, that is to say, with the temperature), the phenomena observed after stimulation and also without stimulation become very different. First of all, while in the first days after the section of the nerve, or in the first hours after death, the skin is completely pale, as well as motionless. Later on it begins to show some coloration, and we notice on it the wonderful phenomenon of the so-called cloud-migration (Wolken- or Wellen-wandern), which may be described as an almost continuous and uninterrupted wandering from one spot to the other of a condition of expansion of the chromatophores, that is to say, of contraction of their radial fibers. It is characteristic of this phenomenon that the contraction, during its wandering, never jumps; it always goes from one chromatophore to the next, and never to one further away. This fact is of the utmost importance for the explanation of this phenomenon, which is completely different in its nature from anything we may observe on the normally innervated skin. In the latter case you may now and then observe something superficially resembling the phenomenon described above, but in this case the expansion appears here and there in completely separated territories, which, if the observation is accurate enough, may be recognized as those innervated by the same nerve, or group of nerves. In this case the contraction of the radial fibers is the result of an excitation of one or more nerves; in the first one we have to do with a purely muscular phenomenon, as we shall very soon see.

As a matter of fact, if a stimulation is sent to the skin on which the "cloud-migration" is observed, either through the nerves or directly, the phenomena produced are very different from those normally observed. No effect whatever follows the stimulation of the nerves. When the electric stimulus is applied directly to the skin, we notice that the excitability of the chromatophores, to galvanic as well as to faradic currents, is very low. It is only with very strong currents and with very prolonged stimulations, that it is possible to get an effect. Even in this case the expansion is limited just to the few chromatophores lying between the electrodes. It starts only after a prolonged delay (often after the stimulation is over) and lasts longer than the stimulus. This effect is not comparable to the one observed on the normal skin, where the direct application of any stimulus produces an immediate and diffuse expansion of the chromatophores which lasts just as long as the stimulation itself. The electric excitability in the two conditions is very different. On the other hand, in the skin where this modification of the electric excitability occurs, the mechanical excitability of the radial fibers of the chromatophores is very high, much higher than when the innervation is intact. This augmentation is accompanied by an increase of the excitability for luminous and perhaps also for chemical stimuli. This increase of the mechanical excitability makes possible the explanation of the phenomenon of the cloud-migration. If the skin is left unexcited and in the dark, its chromatophores are at rest and it is pale, just like the normal skin. As soon, however, as a stimulus reaches any spot on the skin, the chromatophores of this spot expand; their radial fibers, it follows, contract. By their contraction, however, the fibers pull on the next ones; and as these are in a condition of mechanical hyperexcitability, this very slight stretching represents a stimulation strong enough to produce the contraction of the stretched fibers. In this way the contraction proceeds, giving the impression of a "wandering cloud." The same explanation makes it clear why, when any spot in the denervated skin is stimulated, the contraction very often propagates in concentric circles, or at least in the two opposite directions. On the other hand, it might seem strange that the contraction advances, that is, that it only goes one way and not back. It must be considered, however, that the radial fibers, like every excitable tissue, have a refractory period. The pull exerted by the fibers of the chromatophore which contracts, first reaches all surrounding fibers which are at rest, and therefore provokes their contraction. This contraction, like that of the fibers of the previous chromatophore, also exerts a pull, and therefore a stimulation, all round; but while on one side this stimulation reaches fibers which are at rest, on the other it reaches the fibers of the first chromatophore, which have just undergone their contraction and are therefore in the refractory period following the excitation. The stimulation given by the pull is therefore ineffective on these fibers and the expansion of the chromatophores extends always only to one side and not to the other.

The chromatophores of the skin which has been for some time deprived of its nervous connection are, as we have seen, the bearers of very remarkable properties, which are, at least apparently, quite different from those of the normally innervated chromatophores. The obvious explanation of this fact is that, after the section of the nerve, the latter degenerates, and the radial fibers, deprived of this control, develop some new properties of their own. There are two more facts worth noticing in this connection. The first is the rapidity of the degeneration of the nerves after the section. Even in the coldest season, it never takes more than a week or ten days for the nerves to degenerate completely in *Octopus* and *Eledone*. The physiological results, which are in themselves very clear and conclusive (Sereni, 1929*c*), have been, at my suggestion, controlled by Young (1929) with histological examination. In the last few days I have been able to perform the same experiments here on *Loligo*, and I have been able to see that in this much more delicate animal the physiological aspects of the degeneration of the nerves are complete (in summer, at least) in not much more than twenty-four hours. After this time it also becomes impossible to stain the nerves going to the chromatophores with methylene blue. In this instance, as in those previously quoted, the physiological results are perfectly checked by the morphological ones. When one thinks of the length of time required for nerve-degeneration in the frog, there is hardly need to emphasize the difference between the two. This shows, I think, that the rapidity of the degeneration of the nerve after its section cannot be simply dependent upon the body temperature. On the other hand, when the nerve degeneration is complete, no more changes occur in the muscles, even after six to seven months.

The second point I want to emphasize is that almost exactly the same changes occur after the death of the animal as after the section of the nerves. The most outstanding difference (which helps to explain other ones) is that, in the former case, the changes occur much more rapidly—in the hot season in a few hours. It is very difficult to admit that, during this short time, great changes may occur in the muscle-fibers themselves, of the kind observed after the denervation of muscles in vertebrates. Since, however, the phenomena observed in the chromatophores and in the muscles of the cephalopods are exactly the same both after denervation and death, it is only left us to conclude that in neither case does "degeneration" of the muscle-fiber occur. The phenomena observed are much more those inherent in the muscle-fibers as such, unaffected by the nerves.

It is therefore on the skin of an animal whose nerves have been cut some days before, or which has been dead for some hours, that we must perform our experiments when we want to consider the chromatophores from the standpoint of the physiology of muscle-fibers. The radial fibers are smooth fibers, but in the rapidity of their contraction and relaxation they approach striated fibers. On the other hand, in their notable capacity for rhythmical contraction they resemble the fibers of the myocardium. Their study is therefore of much interest, the more so because when you experiment on the chromatophores, you are in reality doing many experiments at once. As a matter of fact, since every fiber contracts independently, the effect noticed is not, as in every other muscle, a summation of the individual effects of each fiber, but more like a statistical average of them. The result is, therefore, more likely to approximate the truth.

It is easy to observe in the chromatophores many phenomena regarding the general physiology of muscles. Cadaveric rigidity, for instance, reveals itself with an extreme expansion, which lasts for some hours and passes off slowly. It is preceded, during some hours, by a beautiful and very irregular play of colour.[2]

In addition, it is possible to study the action of many factors on the chromatophores, with the advantage, already emphasized, that you can be sure that the effect observed is due to an action on the muscle-fibers themselves. I have tried the effect of high and low temperature and of changes in the osmotic pressure (Sereni, 1927). There are beautiful researches by Hertel (1907) on the action of lights of different wave-length which affect differently the various kinds of chromatophores. By the way, since the colour belongs to the chromatophores proper and not to the radial fibers, this fact shows that between the former and the latter, relations exist which cannot be nervous, since the experiments of Hertel were performed on paralyzed skin.

Among the advantages of the chromatophores in comparison with other objects for the study of muscular physiology, one of the more important is that, since the fibers are isolated and very superficial, they are all reached and affected practically at the same time. Consequently there is no lagging behind. This advantage is especially noteworthy in the experimental analysis of the action of chemical substances. I have tried the action of different cations and anions (Sereni, 1927). All of these, in solutions isotonic with sea water, induce a more or less intense and rapid contraction of the radial fibers. Among the monovalent cations the most active is K, the least Na; between these two extremes other cations act as follows:

$$K > Rb > NH_4 > Cs > Li > Na.$$

Ba is the most active of bivalent cations; the others are ordered as follows:

$$Ba > Sr > Ca > Mg.$$

While Na produces a very distinct contraction, Mg has only a short and indistinct action in this sense, and its chief effect is to produce a complete but reversible paralysis. It is Mg which antagonizes both the K and Ca in sea water in regard to their action on the chromatophores, and no solution without Mg is able to maintain the latter in good working condition for any length of time.

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[2] This phenomenon was demonstrated by means of cinematograph film.

Among the anions the series, which is less distinct, is as follows:

$$SO_4 > HCO_3 > I,\ SCN > NO_3,\ Br > Cl.$$

It would take us too far to compare these series with the similar ones obtained on other objects and especially on smooth and striated muscles, and we must be satisfied with this simple hint.

The majority of these ions produce exactly the same effect on the normal and denervated skin. This means that they act on the muscle-fiber proper. Since they act also when the fibers have lost their direct excitability, they probably act on the contractile mechanisms themselves. While the ions act peripherally, none of the drugs I have tried, with the probable exception of histamine, act at all under these conditions; we can therefore conclude that they do not affect the contractile mechanisms, on which the ions act. If we experiment with the same drugs on skin which has been denervated for a very long time, only a few of them, namely strophanthin and digitalin, show their action, which consists in a powerful and very durable contracture. Since, under these conditions, the radial as well as the other muscular fibers have lost their indirect, but still retain their direct excitability, the conclusion is that strophanthin and digitalin act on the excitatory apparatus of the muscle-fiber.

On the contrary, on the normal, freshly isolated skin it is possible to evoke an intense expansion of the chromatophores (that is to say, a contracture of their radial fibers) not only by different ions or by strophanthin, but also by many other drugs, such as nicotin, acetylcholine, choline, pilocarpine, arecoline, coniine, etc. In the normal skin many nerves and even nervous plexi (without ganglion cells) are to be found; and it seems at first very likely that, since the last-named drugs do not act directly on the muscle-fibers, they evoke the contraction of the latter through a stimulation of the nerves. It is possible, with a little skill, to isolate a fairly long piece of some nerves, for instance, of the coloration nerves of the tentacles or of the mantle-connectives. If now any of the above-named drugs, say nicotine, is allowed to act on this isolated nerve, but not on the chromatophores, no contracture occurs. The nerve may or may not lose its excitability after more or less time, but there is no sign of any stimulation. It seems highly improbable that the action of drugs on different parts of the nerve-fibers is different. It appears, therefore, safe to conclude that nicotine and its fellow-drugs do not produce the contracture by acting on the nervous plexi of the skin. There is another fact which makes this conclusion quite certain. We have seen above that the degeneration of nerves begins after a very short time and is complete a few days after the section. Now, after this delay, while the indirect excitability of the chromatophores is completely and forever lost, nicotine still maintains the capacity for producing an intense contracture. The seat of its action cannot, therefore, be in any part of the nerve, which at this time is completely out of function, but must be more peripheral. On the other hand, we have seen that nicotine and other similarly acting drugs do not act on the muscle-fibers proper.

There is only one solution to this puzzle, namely, that these drugs act neither on the nerve nor on the muscle-fiber, but on something intermediate, on something which does not belong to the nerve, because it does not degenerate with it, but is strictly dependent on it for its function. This dependence is shown by the fact that when the nerve has degenerated, the excitability of the muscle-fiber for electrical and mechanical stimulations is the same in the first months, when the intermediate structure is still there, as later, when it also disappears. The excitability for nicotine and other specific chemical stimulants, which is still present in the first period after the nerve-degeneration, disappears after some months. It is just this difference which allows the conclusion that between the two periods there must be a change, namely, the presence or absence of the intermediate zone on which nicotine acts (Sereni, 1930).

There is hardly need to recall how far these facts correspond to the well-known phenomena which occur after nerve-section in the vertebrates. They are the more interesting because they represent the first experimental examination of these problems outside the vertebrates. As in cephalopods, also in vertebrates, nicotine acts on a part intermediate between nerve and muscle, a part which does not degenerate with the nerve but disappears only much later, very likely, as in cephalopods, because of a sort of *atrophia ex non usu.*

There is, however, a difficulty in connecting and comparing the phenomena in vertebrates and in cephalopods. In the first group the study of these phenomena is very much helped by the existence, in curari, of a drug which antagonizes the action of nicotine and paralyzes the motor nerve-endings. Now, as mentioned above, curari does not show its specific action in the cephalopods at all, nor does it in any way interfere with the action of nicotine. It was just because of this failing of curari that up to the present nothing was known about the connections between nerve and muscle in cephalopods.

It must be added that so far the most painstaking examination, in the hands of such careful workers as Hofmann (whose results in this regard I can fully substantiate), has not succeeded in showing any differentiation of the motor nerve-endings in the cephalopods of the type of motor end-plates. It is on these latter, however, that curari most probably acts in the vertebrates, and their absence is in good accord with the absence of action of curari.

On the other hand, it must not be believed that, because curari fails to show its characteristic action, it is impossible to find in cephalopods any drug which specifically paralyzes the same zone which is stimulated by nicotine. By a systematic experimental investigation of the action of many drugs on the muscles of cephalopods, I was able (*loc. cit.*) to find that strychnine more characteristically and specifically, but also quinine, caffein, ephedrine, tetrahydrobetanaphthylamine, act on the intermediate zone, which they paralyze, antagonizing in this way the action of nicotine and of the similarly acting drugs.

Things seem to become rather complicated, I fear; and complicated, indeed, they are. A possible key is given by the most recent work of Riesser (1925) on the vertebrates. This author has shown that, contrary to the opinion of Langley, it is quite possible to distinguish the action of curari as an antagonizer to nicotine from the block of the conduction of impulses from nerve to muscle. The latter, which appears for minor doses, disappears after the degeneration of the nerve, and must be located in the nerve-ending proper. The former effect, which can be demonstrated a long time after the section of the nerve, although in the end it also disappears, has as its seat a more peripheral part, the receptive substance of Langley.

If we compare these results with our own in the cephalopods, we must conclude that the character of the differentiation we succeeded in demonstrating in the radial fibers as well as in all other muscle-fibers of cephalopods is similar, although not identical, to that of the receptive substance in the muscles of vertebrates. It cannot be said whether the physiological and pharmacological differentiation—the only one demonstrated by our results—corresponds to any morphological differentiation. That this last has not yet been found cannot be accepted as a definite negative proof, since, according to the opinion of Boeke, the morphological structure corresponding to the receptive substance should be his periterminal network, and it is quite possible that it has not yet been demonstrated because of technical difficulties. On the other hand, there are neither physiological nor pharmacological proofs of a differentiation in the cephalopods of the motor nerve-endings, corresponding to that on which curari acts.

This is in accord with the results of the histological research, which fails to demonstrate any form of end-plates (which could not be overlooked).

We have described the changes in the excitability of the chromatophores which follow the section of their nerves. We have not, however, or only too briefly, referred to the fact that, soon after this section, the chromatophores expand spontaneously, developing what has been called a "peripheral autotonus."

It is too late now to describe at length this phenomenon and to discuss its origin. I should only like to recall that betaine, which, as stated above, is to be found in the cephalopods, acts on their chromatophores exactly like nicotine. On the other hand, tyramine, whose presence and action on the nerve-centers has been described before, very much increases the excitability of the radial fibers of the chromatophores. The action of these two substances suffices, therefore, to explain the phenomena (expansion, increase of the mechanical excitability) observed on the chromatophores after the section of their nerves. As they are to be found in the body and the blood of the cephalopods, it seems not too difficult to admit that they play a part in the production of these phenomena. Therefore, while on one hand they control the behaviour of the chromatophores through their nerve-centers, on the other they act on the chromatophores themselves, but this peripheral influence can only be shown when the central control has ceased to act for some time.

Ladies and gentlemen: At the beginning of this lecture, I told you that the study of the chromatophores of the cephalopods has many interesting aspects, and that they can be examined both for themselves, when their radial fibers supply us with an almost unique opportunity of studying under the best experimental conditions the reactions of physiologically isolated muscle-fibers, and as indicators of phenomena taking place in their ruling centers.

I hope that the results presented may have substantiated these claims. To summarize them in a few lines, we can say:

1. The chromatophores, under normal conditions, are ruled by different groups of nervous centers, both excitatory and inhibitory.
2. These centers are under the continuous control of at least two humoral factors, whose action determines the "tonus" of the nerve-centers, and by this means, the condition of the chromatophores themselves. This is the first demonstration of humoral correlations in cephalopods, and it is very likely that it is connected with an endocrine function of the posterior salivary glands.

3. The connection between nerve and muscle has for the first time been studied in cephalopods, and I was fortunate enough to show that there is a differentiation, whose characteristics are similar to those of the receptive substance in the vertebrates. There seems to be no differentiation corresponding to that on which curari acts.

These are only a few instances of the many experiments for which the chromatophores of the cephalopods offer an admirable object. I wonder if somebody may perhaps ask what is the purpose of repeating on different objects experiments already performed. It may be that this is sometimes an idle and useless task; although, you must remember, it has happened more than once that repetition of former experiments on a new object has led to quite new results, not infrequently more important than the first ones. But I daresay there is one more reason, and a very good one, for welcoming every addition to the number of our experimental animals.

Physiology today is moving in the direction of general physiology. Now we are perhaps, all of us, too often inclined to forget that a general physiology cannot be only a general physiology of vertebrates, but has as its necessary base and complement comparative physiology. On the other hand, to compare the physiology of different animals, we must study the physiology of each of them. To say it in different words, general and comparative physiology can only rest on an ever-widening knowledge of the special physiology of the different types. It often happens that this widening is accompanied by an increase and a deepening as well; if not, the results will be welcome just the same.

It may appear that I am rather too keen on this point. I hope, however, that this will not be your opinion, ladies and gentlemen, who have gathered from all over the world at this place, which is, undoubtedly, together with its sister Station in Naples, the chief shrine where comparative physiology and general physiology have been honored and practiced daily for many years and, *favente Deo*, will be for many years to come.

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THE EFFECT OF CARBON MONOXIDE AND OF HYDROGEN SULPHIDE ON NERVE IRRITABILITY

FRANCIS O. SCHMITT AND LYLE V. BECK

The Department of Zoölogy, Washington University, St. Louis, Mo.

Indirect evidence has been accumulating which indicates that the conduction of the impulse in nerve depends in some way upon phenomena of oxidation. The purpose of the experiments to be reported in this preliminary paper is to determine, if possible, whether the iron-containing respiratory enzyme of Warburg is required for the activation of the oxygen used in the production and propagation of the nerve impulse. For this purpose we have adopted the procedure of treating the tissue with substances which unite with the respiratory enzyme and hence act as poisoners of this oxidative system in the cell. Warburg (1927) has shown that carbon monoxide may inhibit cellular respiration very completely in the absence of light and that this inhibition is reversible to a considerable extent by illumination. Similar effects are produced by hydrogen sulphide although the complex formed by the union of hydrogen sulphide with the respiratory enzyme is not sensitive to light (Negelein, 1925).

EXPERIMENTAL

Nerve irritability was followed by determining the threshold shock required to produce a muscle twitch. Nerve-muscle preparations from green frogs were used, the customary arrangement being as shown in Fig. 1. The nerve passed through two chambers, *A* and *B*, each containing a pair of platinum stimulating electrodes and an inlet and an outlet tube for the passage of gases. It was also possible to immerse the nerve in different solutions in each chamber if so desired. Condenser discharges were used for stimulation. Fig. 2 illustrates the electrical arrangement employed for this purpose. As shown in the diagram, when the key was up the condenser was being charged to a potential indicated on the voltmeter. Depression of the key discharged the condenser through the nerve. A selector switch facilitated shifting from one condenser to another. The choice of the condenser value was determined by the relative irritability of the preparation at the beginning of the experiment. In most cases a 0.005 mfd. condenser was used and the threshold at the outset was

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usually less than one volt. Having once determined upon a condenser value to be used in any particular experiment, this value was used throughout the experiment.

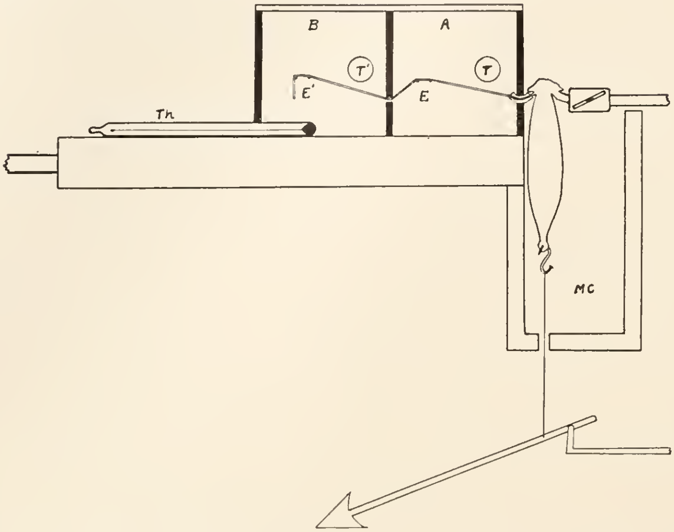

FIG. 1. Sketch of nerve-muscle preparation in place. Nerve lies over pair of electrodes *E* in chamber *A*, and *E'* in chamber *B*. *T* and *T'*, inlet tubes for passage of gas; *Th*, thermometer; *MC*, moist chamber for muscle.

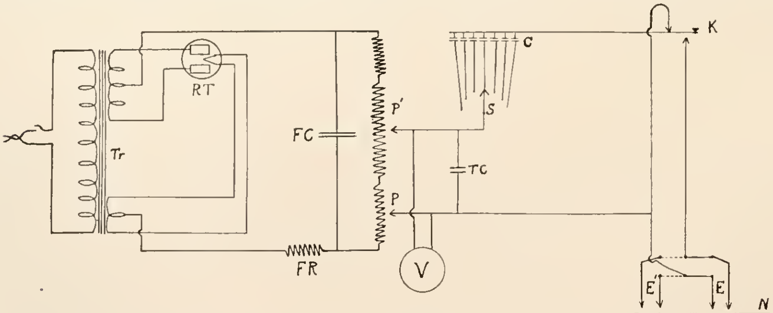

FIG. 2. Electrical diagram for stimulating device designed to operate on alternating current. *C* represents a series of condensers varying in capacity from 0.001 mfd. to 0.1 mfd. *E* and *E'* are pairs of platinum electrodes in chambers *A* and *B*, respectively. *FC*, 2 mfd. filter condenser; *FR*, filter resistance; *K*, key; *N*, nerve; *P*, potentiometer, 1000 ohms; *P'*, potentiometer, 10,000 ohms; *RT*, rectifier tube UX 280; *S*, selector switch; *Tr*, transformer; *TC*, 2 mfd. tank condenser; *V*, multiscale voltmeter.

THE EFFECT OF CARBON MONOXIDE ON NERVE IRRITABILITY

The carbon monoxide was generated by dropping concentrated formic acid into hot sulphuric acid. In experiments in which pure carbon monoxide was used, the gas was passed from the generator over glowing copper through wash bottles to the preparation through sealed tubes. When the carbon monoxide was to be diluted with oxygen it was passed from the generator into large calibrated bottles and the proper dilution with oxygen made. After allowing some time for diffusion of the gases, and having analyzed samples, the gas mixture was passed through wash bottles to the preparation. Some difficulty was encountered in preventing gas leaks from the outside into chamber *A*, and from chamber *A* into chamber *B*. To prevent this, a very slow stream of moist gas was passed through each chamber for the entire duration of the experiment. In experiments in which carbon monoxide was passed through one chamber and nitrogen through the other, the pressure of the carbon monoxide was maintained at a somewhat higher level than that of the nitrogen. An arc light served as a source of illumination and the rays were passed through copper sulphate to filter out the long wave lengths. A thermometer lying next to the nerve in the chamber indicated the temperature.

It was found that nerves lose their irritability fairly rapidly in mixtures of carbon monoxide and oxygen in which the concentration ratio is 20 to 1 or greater, although failure in pure carbon monoxide does not appear to be more rapid than in pure nitrogen. While it was possible to determine irritability quite accurately by the present method, it would perhaps have been more satisfactory to have used the action potential as the criterion of the reactivity of the nerve.[1] Under the conditions of these experiments it was possible for all but a few of the nerve fibers to have failed completely without greatly affecting the threshold readings. Consequently the usual result was that treatment of the nerve with carbon monoxide had little effect on the threshold readings until the last fibers began to fail, at which time the threshold rose rapidly and reached infinity in a short time. This greatly restricts the period of time during which one might expect to obtain an effect with illumination. Nevertheless, in a number of experiments it was found that illumination of nerves during the period of failure in mixtures of carbon monoxide and oxygen caused a definite decrease in the threshold (see Fig. 3). The threshold usually continued to decrease as long as the nerve was illuminated. Numerous instances were recorded in which illumination caused a return of irritability in nerves which had become completely non-irritable in carbon monoxide mixtures; that is, the threshold returned from infinity to definitely readable values (100–200 volts with a 0.005 mfd.

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[1] This has now been accomplished by means of the cathode ray oscillograph and will be published shortly by one of us.

condenser). In some cases a definite after-effect of the light was noted which lasted for some minutes, but, owing to the limitations of the threshold method, investigation of this effect must be deferred.

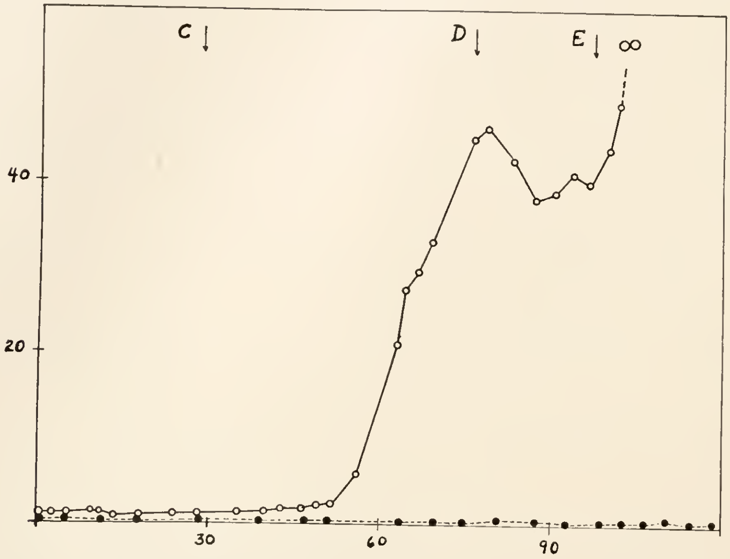

FIG. 3. Effect of carbon monoxide on nerve irritability. Ordinates, threshold in volts (with a 0.001 mfd. condenser); abscissae, time in minutes. Circles represent thresholds of portion of nerve in chamber *B*; points, in chamber *A*. At *C*, a mixture containing about 99.5 per cent carbon monoxide and 0.5 per cent oxygen was passed into chamber *B*, air being passed through chamber *A*. At *D*, nerve in chambers *A* and *B* was illuminated; at *E*, illumination was discontinued.

Although the most striking light effects were observed in nerves which were failing in carbon monoxide-oxygen mixtures, similar results were also obtained when carbon monoxide carefully freed of oxygen was used. It seems probable that these results are best explained on the assumption that traces of oxygen leaked into the chamber through the tube which conveyed the nerve and that this small amount of oxygen sufficed to unite with the iron catalyst under the influence of light, causing a temporary partial return of the irritability.

THE EFFECT OF HYDROGEN SULPHIDE ON NERVE IRRITABILITY

To test the effect of hydrogen sulphide on nerve function, two types of experiments were performed. In the first type the nerve was freely suspended over the electrodes in both chambers and hydrogen sulphide from a generator was passed into chamber *B*, air being circulated through chamber *A*. In the second type of experiment the nerve was made to dip into a solution containing hydrogen sulphide in chamber *B*, but was freely suspended over the electrodes in chamber *A*. In the latter case the solution was made by dissolving sodium sulphide in Ringer solution and adjusting the pH to 7.6 by the addition of hydrochloric acid. It was possible by this method to replenish the sulphide solution frequently, thus preventing escape of gas and corresponding dilution of the solution. Fig. 4 illustrates typical results obtained in these experiments. The threshold of the portion of the nerve treated with hydrogen sulphide was found to rise slowly for a period of time and then to rise very rapidly. The threshold of the control stretch of nerve in air remained unaffected. Recovery in Ringer solution was usually incomplete. Replacing the sulphide-containing solution with Ringer solution produced scarcely any recovery of the nerve; it was usually necessary to withdraw the nerve from the chamber and bathe the whole preparation in Ringer solution for a time. This was doubtless due to the fact that during the period of poisoning, hydrogen sulphide diffused into the nerve a considerable distance beyond the point of immersion in the solution. Subsequent replacement of this solution with Ringer solution had little effect on the portion of the nerve poisoned by the diffused hydrogen

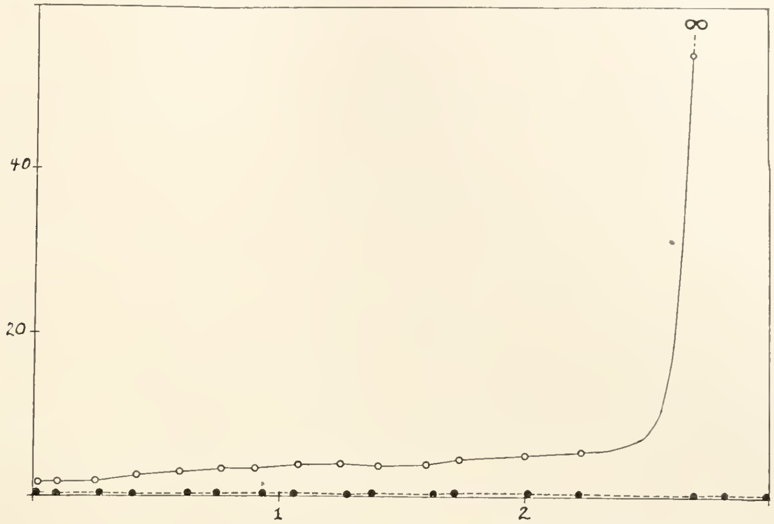

Fig. 4. Effect of M/500 solution of sodium sulphide (adjusted to pH 7.6) in Ringer solution on nerve threshold. Ordinates, threshold in volts (with a 0.005 mfd. condenser); abscissae, time in hours. Circles represent thresholds of the portion of the nerve dipping in sulphide solution in chamber *B*; points represent thresholds of control stretch of nerve in chamber *A*.

sulphide. Hence to obtain recovery it was necessary to remove the preparation and bathe it in Ringer solution to remove this remaining sulphide.

DISCUSSION

Although the present experiments are subject to certain limitations inherent in the threshold method, as pointed out above, the data demonstrate that both carbon monoxide and hydrogen sulphide may render the nerve non-irritable. The effects produced by carbon monoxide were in almost all cases reversible, those produced by hydrogen sulphide were less reversible. Since these substances are efficient poisoners of the iron-containing respiratory enzyme, it is likely that the oxygen which is used in the production and propagation of the impulse has first to be activated by the respiratory enzyme. This view is supported by the fact that illumination of nerves failing in carbon monoxide-oxygen mixtures causes a temporary return of irritability. There is also ample evidence indicating that this light effect is not due simply to a rise in temperature. The rise in temperature as recorded by the thermometer lying next to the nerve seldom exceeded 1.0–1.5° C. for a ten minute period of illumination. Furthermore, light had no effect on an equally illuminated portion of the same nerve in the chamber immediately adjoining the carbon monoxide treated portion. Complete discussion of the mechanism of this catalytic oxidative system and its possible relation to the irritable mechanism in nerve must be deferred until the completion of further experiments now in progress, in which the action potential has been measured by means of the cathode ray oscillograph.

SUMMARY

1. Both carbon monoxide and hydrogen sulphide, which presumably inhibit cellular oxidations by combining with the iron-containing respiratory enzyme, render nerve non-irritable. The effects of carbon monoxide appear to be completely reversible, those of hydrogen sulphide, less so.

2. Illumination of nerves failing in mixtures of carbon monoxide and oxygen causes a temporary partial return of irritability and there is some evidence that there is an after effect of the illumination which may last some minutes.

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PARAMECIUM INFUSION HISTORIES

I. HYDROGEN ION CHANGES IN HAY AND HAY-FLOUR INFUSIONS

EDGAR P. JONES

DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF PITTSBURGH

The data presented in the following article were collected in the course of observations made upon a series of forty-seven infusions, in an effort to accumulate as complete a record as possible of the events which take place in a culture of *Paramecium multimicronucleatum*. This article will be restricted to pH behavior. Other phases of the culture history will be dealt with in articles to follow.

HISTORICAL

The literature of this subject is quite limited.

Bodine (1921), using colorimetric methods, studied the hydrogen ion behavior of various infusions in which he was able to raise mixed protozoan cultures. His infusions passed through acid and alkaline cycles in a manner which closely resembled the behavior of the infusions that we are reporting as types, with the difference that the various cycles were retarded in his infusions. This was probably due to the larger volumes of the infusions which he studied. Our large cultures displayed a similar retardation.

Pruthi (1927) studied the hydrogen ion concentration of a series of hay infusions of different sizes which were made without boiling, and which were allowed to develop protozoan populations without seeding. In these studies he recorded changes of pH which were quite similar to those which are reported in this paper.

Darby (1929 and 1930) has demonstrated in a very convincing manner that the division rate of *Paramecium caudatum* and *P. aurelia* is greatest at a pH slightly less than 7.00, and that an increase or decrease of hydrogen ion concentration from this optimum will reduce the division rate.

Beers (1927) has reported the hydrogen ion concentrations of cultures of bacteria alone, and of bacteria and *Paramecia*. He says, "It is seen that the hydrogen-ion concentration of fresh infusion was pH 6.2, and that it increased to 5.8 during the first twenty-four hours, after which it steadily decreased as the infusions grew older. This succession of changes in hydrogen-ion concentration was observed regularly in all infusions prepared." It will be noted that his results agree only in a very general way with ours, both with respect to the pH variation and the time of greatest hydrogen ion concentration.

Phillips (1922), experimenting on the feeding of known kinds of bacteria to *Paramecium*, reported at the termination of her work that two of her cultures showed a pH of 8.2, while the third had a pH of 8.4.

Johnson (1929) reported that the cultures from which he took *Paramecium* ranged between pH 7.4 and 8.0. He described this stage as one in which the *Paramecia* were scattered throughout the culture.

Beers (1927), while investigating the encystment of *Didinium* in buffered solutions of varying hydrogen ion concentration, found that the limits within which the organism could live were approximately pH 5.0 to pH 9.6. Crane (1921) found practically the same limits for *Paramecium*, and Johnson (1929) determined that *Paramecia*, in drops of solution tested colorimetrically, were killed at pH 5.0. His electrometric readings indicated a pH somewhat lower.

Materials and Methods

This series of studies was made on infusions of three sizes. The medium size, 700 cubic centimeters, was considered the standard, and the major portion of the cultures contained that amount of liquid when made. Three infusions of one third that size were studied, as were three of 7 litres. The 700 cc. cultures were all kept in containers of uniform size and shape. In the discussion which follows, only the 700 cc. cultures will be considered, unless the larger or smaller infusions are mentioned specifically.

Of the infusions studied, some were loosely covered, others were open. Experience proved that the loosely covered jars lost two cubic centimeters by evaporation daily, while the open jars lost six times that amount. This uniformity of evaporation rate was obtained by keeping all of the cultures in a constant temperature room at 27° C.

The standard culture of 700 cc. capacity became divided into a considerable number of groups, dependent upon the quantities of hay and flour that were used in preparing them. The practice was to prepare at least three infusions of any one of the more important types simultaneously, and the facts so obtained were again checked in some cases by preparing a single infusion of the same kind later. The composition of these infusions is most readily described through the agency of the table which follows.

TABLE I

Infusions in which Paramecium Lived and Multiplied

No. Infusions Made	Grams of Hay Used	Grams of Flour Used	Covered or Open
9	1	0.1	Covered
6	1	0.	"
1	2	0.	"
1	4	0.	"
4	1	0.1	Open
4	1	0.	"

Infusions in which Paramecium did not Live

No. Infusions Made	Grams of Hay Used	Grams of Flour Used	Covered or Open
4	4	0.4	Covered
1	4	0.1	"
1	2	0.2	"
2	8	0.8	"

All infusions were prepared by bringing distilled water to a boil in a covered granite kettle, adding the required amount of timothy hay, and boiling for ten minutes. The uniformity of the hay for the entire experiment was controlled by passing a considerable amount of the original hay through a meat chopper, and then thoroughly mixing the cut product. White wheat flour, when needed, was mixed into the hay and added to the infusion with it.

Infusions so prepared were poured into their respective containers and allowed to cool until the following day, when they were seeded with two hundred *Paramecium multimicronucleatum* of a pure line. This formula is essentially similar to the one used by Packard which was on file in this laboratory.

Hydrogen ion concentration measurements were made at frequent intervals during the life of the culture, as were population counts. These data were plotted against time in a series of graphs. This method proved convenient, as it showed clearly the relationships existing between population per cc., volume of infusion remaining, and hydrogen ion concentration on any day of the infusion life.

The pH readings were made with a No. 5270 Youden Apparatus.

All infusions were stirred violently at intervals of one to three days, when population counts were made.

Observations

From the data gathered, certain facts concerning the hydrogen ion concentration of a hay, or of a hay-flour culture of *Paramecium multimicronucleatum* stand out with such clearness, and are so typical of the results obtained in this experiment, that it seems safe to illustrate the general hydrogen ion history of a successful culture by contrasting one culture which failed, probably due to the prolonging of the acid period, with a second one which succeeded. We presume that this success was due to a more favorable pH behavior on the part of the second culture. Culture 36, of which Plate 1 is the graphical record, made with one gram of hay and 0.1 gram of white flour, developed a culture in a normal and satisfactory manner. It had a pH of 6.93 within an hour of the time that it was made, and while it was still slightly warm. During the next twenty-four hours, it went down to pH 6.08, at which time it was seeded with two hundred *Paramecia*. For some reason it seemed to hesitate in its pH reducing process. This is the usual behavior. The second day found it still pH 6.09 at 10 A.M., but the evening of the same day showed a pH of 5.64. After midnight of the third day, when the pH was 5.15, it reached its turning point, for by 11 A.M. of the fourth day the reading was 5.22. Midnight of the fourth day showed pH 5.38, and from this point it made a rapid return to pH 6.95, the hydrogen ion concentration which it reached by midnight of the eighth day.

The hydrogen ion behavior of this culture is, essentially, the behavior of each culture in the series of experiments in which the *Paramecia* survived the extremely acid period. In most of the cultures the highest hydrogen ion concentration noted was between pH 5.00 and 5.50, although culture 35 developed a pH of 4.83 without killing all of its 200 seed *Paramecia*. Such an acid condition was usually developed in about four days, and a condition of approximate neutrality was reattained in from eight to ten days. The following ten days characteristically fluctuated between slight acidity and slight alkalinity, following which there was a gradually increasing alkalinity throughout the remainder of the life of the infusion. This final alkalinity seldom was more than pH 7.5 when 250 cc. of the infusion remained, and usually it was less.

In the open cultures, where a greater reduction of the volume took place, a rising pH resulted. Culture 4, the graph of which is not shown, recorded a pH of 8.31 when only 20 cc. of the original 700 remained. Infusions such as No. 4, which had lost a greater portion of the original fluid through evaporation, usually contained 2000 or more *Paramecia* in each cc. of the culture. Such remarkable concentrations of the

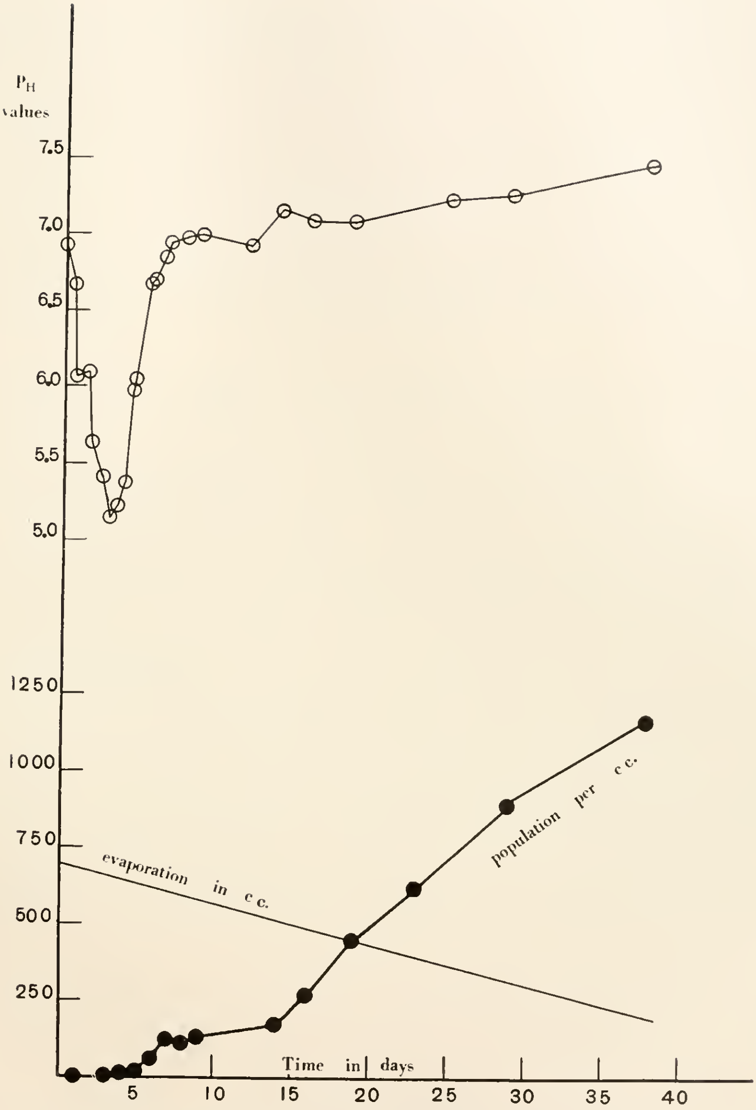

Plate 1. This graph shows the typical hydrogen ion behaviour of a hay-flour infusion which was seeded with 200 *Paramecium multimicronucleatum* before it had reached its maximum acidity.

This pH record is typical of all cultures in this series of experiments, in which the *Paramecia* lived.

Paramecium population might be continued for ten days or more, if open jars, which had evaporated the greater part of their infusion, were covered.

It was repeatedly noticed that the *Paramecium* population in a successful culture was concentrated near the top in a younger infusion; was uniformly distributed throughout the container in a culture of medium age; and finally, that the animals concentrated at the bottom of the culture. It seems probable that this systematic behavior is associated with the changing pH conditions that are reported in this paper. Johnson (1929), Child and Deviney (1926), and Pruthi (1927) have reported somewhat similar observations of population distribution.

Such a distribution of animals did not appear to be caused by differences in hydrogen ion concentration at different levels of the infusion. The electrometric readings of the pH of material taken from the top, middle, and bottom of a container were alike when investigated on different occasions. Color indicators added to cultures showed no hydrogen ion concentration differences at the various levels.

Plate 2 is the graphic record of Culture 34. It serves as a contrast to the cultures which were successful. The medium was made by boiling 4 grams of hay and 0.1 gram of flour in the usual amount of water. As is shown in the attached graph, this culture also started at neutrality, hesitated in its pH reducing process when the same quantities of *Paramecia* and infusion were added, after which its pH continued to reduce until it had reached 5.05 by midnight of the third day. If the hydrogen ion concentration had returned to normality in this infusion as it did in Culture 36 which was previously described, it seems probable that a successful culture would have been established. Instead, the pH readings remained between 5.00 and 4.90 for the next five days, at which time an examination showed that all of the animals were dead. The hydrogen ion concentration of this infusion did return to normal a few days later. It was then seeded with a second quota of 200 *Paramecia* and these animals multiplied satisfactorily. The pH when this infusion was seeded the second time was 7.3.

In all such infusions, the list of which is given, it appeared that a prolonged acid phase was induced in the early stages of the infusion by the addition of either too much hay or too much flour. When no flour was used, as much as 4 grams of hay did not induce either superacidity, or acidity too prolonged. The addition of 0.1 gram of flour to such a formula produced a medium which killed. If one tenth of one gram of flour was used, the hay had to be reduced to less than two grams if the seed *Paramecia* were to live.

Such results would have caused us to altogether discontinue the use of flour in the making of the media, had we not noted that the densest populations were secured when flour was used. The choice formula, therefore, came to be one gram of hay and one tenth of one gram of flour in 700 cc. of distilled water. Such a culture in a covered jar would usually support from 300 to 700 animals for each cubic centimeter of the infusion, producing a total population of 200,000 to 500,000.

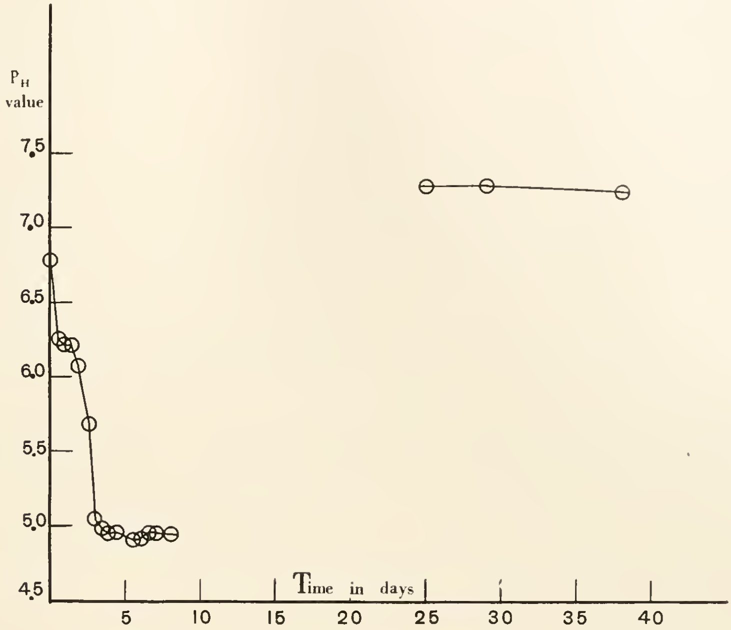

Plate 2. This graph shows the typical pH behavior of a hay-flour infusion which killed its 'seed' population of 200 *Paramecium multimicronucleatum*, presumably as a result of the prolonged superacidity.

No hydrogen ion concentration readings were made between the eighth and the twenty-fifth days. 'Seed' *Paramecia* introduced on the twenty-fifth day grew successfully at the hydrogen ion concentrations shown.

Discussion

A consideration of the data which have been presented indicates that the length of time in which the extremely acid phase continues, determines whether the infusion will kill the seed *Paramecia* which are introduced the second day. This acid phase is accompanied by fermentation, and is evidently brought about by bacterial activity. The degree of hydrogen ion concentration is probably a factor in determining whether all of the seed *Paramecia* will be killed. Crane

(1921) states that pH 5.0 is the greatest hydrogen ion concentration in which *Paramecium* can live for twenty-four hours. Culture 35 of this series developed a pH of 4.83 for a few hours without killing the *Paramecia* in it, but our usual culture did not have a hydrogen ion concentration greater than pH 5.0.

The second stage of the pH behavior, in which the infusion returns to normality and then becomes alkaline, is probably brought about by a second cycle of bacteria. Peters suggested the probability of such bacterial cycles as early as 1907.

Our observation of similar hydrogen ion concentrations at the top, middle, and bottom of cultures would seem to disagree with those of Peters (1907), who found an increase in the titratable acidity as materials were drawn from deeper levels. Fine's (1912) series *A* and *C*, from which he did not strain out the hay, paralleled the behavior of Peters' infusions; but his series *B*, from which he had removed the hay, showed practically no differences in titratable acidity at different levels. Fine believed that this acidity was caused by bacterial action, and he suggested that the greater acidity at or near the bottom was due to the concentration of the bacteria about the hay. Such an explanation might account for the uniformity of our pH readings at different levels, for our cultures were violently stirred at intervals of one to three days, when population counts were made. Such treatment would scatter the bacteria throughout the culture.

We plan to try to determine the species of bacteria which cause these changes; the stages of the infusion in which each bacterium is most numerous; the pH condition which the metabolism of each bacterium induces; and the maximum, minimum, and optimum pH for each. Hargitt and Fray (1917) and Phillips have made a very creditable beginning on bacterial identification and description.

Summary

A study of forty-seven *Paramecium multimicronucleatum* cultures made by boiling varying combinations of timothy hay and wheat flour in distilled water and seeding with 200 pure line *Paramecia* on the second day has yielded the following data:

(*a*) Cultures experienced a changing pH cycle which was invariably quite similar to that shown in Plate 1, if the *Paramecia* lived.

(*b*) The most successful timothy hay-flour medium was made by boiling one gram of hay and one tenth of one gram of flour in 700 cc. of water for ten minutes.

(*c*) *Paramecia* were observed to live in culture media whose pH ranged from 4.83 to 8.31 after the solutions had been violently stirred.

(*d*) As cultures evaporated, the alkalinity increased after the first four days. A hydrogen ion concentration of 8.31 was observed in infusion 4 when 20 cc. remained of the original 700. This infusion had a population of 6000 *Paramecia* to the cc. at that time, or about twelve times the normal dense population.

(*e*) Infusions of 7 liters' volume passed through the same pH cycles, but these cycles were considerably retarded as compared with the 700 cc. infusions.

(*f*) The tendency of infusions to kill the *Paramecium* population was associated with a persistence of the extremely acid condition for a period of several days, as is shown by Plate 2. This prolonged acid condition developed in infusions which were made with too much flour, too much hay, or too much of both ingredients.

(*g*) The radically changing hydrogen ion concentrations which were recorded in successful *Paramecium* cultures were thought to be due at least in part to a changing cycle of bacteria. We plan to try to determine what bacteria are responsible for these changes; the maximum, minimum, and optimum pH for each bacterium, the stages of the infusion in which each bacterium is most numerous; and the pH conditions which the metabolism of each bacterium induces.

(*h*) The extreme concentrations of *Paramecium* which were obtained in cultures which were covered after becoming concentrated by evaporation, where populations of as many as 2000 per cc. were kept for ten days continuously, leads us to question whether excretory matter is as toxic to the organism as has been supposed. It seems probable that the excretory matter becomes broken down and reorganized by bacterial and chemical action before it becomes sufficiently concentrated to injure the animals. It may be possible that in such reactions will be found the explanation of the pH behavior which has been reported.

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GROWTH OF THE THYROID GLAND OF *RANA PIPIENS* IN RELATION TO METAMORPHOSIS

WILLIAM N. ETKIN

(*From the Laboratories of Anatomy, Histology and Embryology, Cornell University, New York and Ithaca*)

An exact study of the relation of the growth of the thyroid gland to metamorphosis is essential to an understanding of this process as well as of the gland itself.

Hoskins and Hoskins (1919) reported no marked growth in the thyroid of the tadpoles of *Rana sylvatica* during metamorphosis. However, Allen in the same year found considerable growth in *Bufo* thyroids at this stage. In studies on *Ambystoma*, Uhlenhuth in 1924 reported a marked growth of the thyroid and a sudden rise in the ratio of the cube root of the weight of the gland (reconstruction) to the body length at this period, followed later by a decrease in this ratio. This diversity in results may probably be due to the difficulty of securing an adequate criterion of growth.

Hoskins and Hoskins (1919) used chiefly methods of histological section and of measurement of the dissected organ but made no quantitative investigation. Allen's (1919) conclusions are based on measurements of fixed, stained and cleared specimens after dissection. In Uhlenhuth's work (1924) wax reconstructions of the glands were weighed.

Serious objection may be offered against all of these methods. In the first place, they fail to differentiate cell proliferation from colloid accumulation, and clearly the interpretation of the results depends to a great extent on this distinction. The methods of Allen and Hoskins and Hoskins are further subject to the criticism that the product of the three diameters of the gland as a measure of its volume is precarious in an organ that changes its shape and compactness as does the thyroid during metamorphosis. Variations in shrinkage that occur in the preparation of the specimen for this method further militate against its success. The method of reconstruction, on the other hand, is so laborious as to render a sufficient accumulation of evidence very difficult. These objections were distinctly realized by Hoskins and Hoskins but not overcome.

The author therefore felt that an adequate quantitative study can not make use of any of these methods. The method adopted in the present study was to count the cells in serially sectioned glands and determine the colloid volume by the use of a planimeter. This is perfectly feasible, since smaller glands involve only a few thousand cells and in larger glands not every section need be counted.

Material and Methods

Tadpoles of *Rana pipiens* were used. All specimens were derived from the same small pond in the fish hatchery near the campus at Ithaca. The identification of the tadpoles was checked by Prof. A. H. Wright.

The tissues were fixed in Bouin's fluid, sectioned serially at 10 μ and stained with hæmatoxylin and eosin.

In taking measurements, the body length of the tadpoles was taken as snout to anus minus the length of the anal canal. This made it comparable to body length in frogs.

In making the cell counts an Edinger projection machine was used, throwing an image of 650 diameters magnification. The colloid masses were first outlined, and using this outline as a guide, the cells could be easily counted. In the larger sections it was found advisable to use a further guide by placing two metal strips upon the image. These could be separated, giving a small boxed-off area for counting. By bringing the upper strip down into contact with the lower and then moving the latter, another area immediately below the preceding could be seen. In this way a large section could be accurately counted. A constant play was maintained upon the fine adjustment of the microscope in order to bring out all planes of the section. In making the counts the general rule was followed that where the sections showed over 500 cells every fifth section was counted; over 250 every third section; over 100 every other section and less than 100 every section. It was found that successive sections were so similar in cell count except at the extreme tips of the gland that this rule gave sufficient accuracy. Successive measurements of colloid mass were not nearly so uniform, which may account for the somewhat greater variations in these figures. It must be said that the colloid was so vague and indefinite in character in some sections of the first stage (stage *H*) as to make an accurate determination impossible. The results for specimen No. 54 were calculated on the basis of only one of the two thyroid lobes, since part of the other lobe had been lost. In the graph the point for this specimen is therefore recorded as a circle.

In making the calculation, the figures for the uncounted sections were interpolated and the whole added. All calculations were made by machine and checked either by repetition on the machine or with a slide rule.

To test the reliability of this method, three determinations were made of the same thyroid lobe. Though for a given section the cell counts or colloid volume varied as much as 15 per cent (average about five per cent), these variations tended to balance out in the result for an entire lobe and therefore the three determinations did not vary by more than 2 per cent for either cell count or colloid volume. This indicates an accuracy well within the normal range of variation.

In determining the dry weights given in Table II, the ordinary chemical procedure was used with the following added precautions. The Stage 2 tadpoles were treated as follows. Their tails were cut off and the bodies dissected in the dried and weighed crucible. The intestine was removed completely, drained into the crucible and laid on paper. By running a needle down for pressure, the entire contents could be removed and the intestine then returned to the crucible. The instruments were wiped on the crucible cover, and the ordinary procedure then followed. Thus only an insignificant amount of tissue fluid was lost and the intestinal contents were eliminated. Although the intestines of Stage 5 animals were found to be empty, they were similarly treated in order to equalize the losses.

RESULTS

Table I shows a summary of results and calculations.

It was found advisable to classify the tadpoles into seven stages which may be characterized as follows:

Stage H: Half-grown tadpoles, body length about 13 mm. and total length about 33 mm. At this stage the thyroid cells occur as small groups somewhat scattered, usually enclosing a colloid mass. Their appearance varies from squamous to low cuboidal.

Stage 1: Beginning of metamorphosis. Body length about 18 mm. Hind legs as buds. Thyroid shows beginnings of definite folliculation. Epithelium squamous to low cuboidal.

Stage 2: Hind legs show growth, are 6–8 mm. in length, body length about 23 mm. Follicular nature of thyroid definitely developed. Epithelium somewhat higher than in Stage 1.

Stage 3: Hind legs about 15–23 mm. Forelegs discernible under operculum. Body about 23 mm. Marked increase in size of thyroid. Epithelium cuboidal.

Stage 4: Hind legs about 26 mm. Forelegs about 10 mm. Body length about 21 mm. Transformation of mouth (and intestine) and resorption of tail taking place. Further growth of thyroid and dis-tension of follicles with colloid. The epithelium generally cuboidal or low columnar. During this stage the tadpole takes no food.

Stage 5: Metamorphosis recently completed, tail stub noticeable. Adult body form. Hind legs about 31 mm. Body somewhat smaller than in Stage 4. The epithelium in the thyroid much reduced in height, generally to low cuboidal or squamous.

Stage P: Post metamorphosis. Hind legs proportionately longer and body much larger than in Stage 5. These showed considerable variation in the height of the thyroid epithelium, some follicles being squamous and others cuboidal.

From Table I it can be seen that the thyroid grows enormously in both cell number and colloid volume during this period. Allen (1919) has assumed that the growth curve of the hind legs is rectilinear, in which case the growth of the gland as given in Table I would be essentially a growth curve of sigmoid form, with the period of most rapid growth in Stage 3 and periods of less activity before and after. It is not necessary, however, to make this assumption to derive the essential significance of these figures, for it can be seen that in proportion to the growth of the animal (as measured by body or hind leg length) the thyroid increases greatly and that the major portion of this increase occurs during the early growth of the hind legs. It is interesting to note that this growth becomes marked coincidently with the sudden growth of the hind legs.

The last column of Table I shows the ratio of the colloid volume to cell count. These figures are interesting in that they show a marked rise during the early stages of metamorphosis and assume a more or less constant ratio in the fourth stage. This might be taken to indicate that the thyroid hormone is not of great importance for the early stages of metamorphosis since it then tends to be stored, or alternatively that the colloid is not simply a storage place for the hormone, but rather takes some active physiological rôle in preparing the hormone for the body. A combination of both factors may operate.

Evidence for the first explanation is found by calculating the ratio of the cell count (or colloid volume) to body length cubed, as is done in the seventh and eighth columns of Table I. By dividing the cell count (or colloid volume) by the body length cubed, we get a measure of the amount of thyroid per unit volume of body.

However, caution must be observed in interpreting these figures, for it is obvious that the body length cubed is a valid measure of the amount of tissue only when the shape, proportions and nature of the tissues remain the same. This condition is fairly well satisfied by the tadpoles until the beginning of Stage 4. In this stage the tail is resorbed and the animal assumes the typical frog shape. This makes it impossible to interpret accurately the figures for this stage.

TABLE I

Preparation No.	Stage of Development	Body Length *mm.*	Hind Leg Length *mm.*	Cell Count	Volume of Colloid mm³. × 10,000	$\frac{\text{Cell Count}}{(\text{body length})^3}$ × 1000	$\frac{\text{Vol. Col.}}{(\text{body length})^3}$ × 1000	$\frac{\text{Vol. Col.}}{\text{cell count}}$ × 1000
43	H	13		3,512	1,542	1,598	702	439
44	H	14		2,062	446	751	162	217
5	1	16		3,320	1,546	812	378	467
1	1	17		3,734	2,311	760	470	618
3	1	18		6,048	2,186	1,037	374	361
16	1	20		7,756	3,528	969	441	455
4	1	22		16,106	17,132	1,513	1,609	1,064
7	2	28	6	18,052	20,974	822	955	1,162
17	2	22½	6	15,482	27,788	1,360	2,441	1,794
48	2	22	6	25,785	21,454	2,420	2,020	832
50	2	23	8	25,756	42,723	2,117	3,511	1,698
8	3	21	14	33,396	59,028	3,606	6,374	1,767
9	3	24	15	38,723	58,434	2,801	4,227	1,509
19	3	24½	17	53,409	78,726	3,633	5,352	1,478
10	3	24	23	64,623	142,346	4,675	10,297	2,202
2	4	19		33,385	72,966	4,867	10,638	2,186
15	4	22	26	49,794	137,162	4,676	12,881	2,755
14	4	22	28	60,779	131,412	5,708	12,341	2,162
11	4	21	28	63,488	143,582	6,855	15,504	2,262
12	5	22	31	59,206	140,540	5,560	13,200	2,378
13	5	21	31	50,306	109,460	5,432	11,819	2,176
21	5	21	31	51,003	147,092	5,507	15,910	2,882
52	5	23	35	72,609	131,737	5,968	10,827	1,814
53	5-P	25	41	42,390	96,507	2,713	6,176	2,277
54	5-P	28	45	46,700	142,431	2,127	6,488	3,050
59	P	32	51	54,809	160,157	1,673	4,888	2,922
60	P	41	70	121,478	267,191	1,762	3,877	2,199

The weight of the animals would be no more satisfactory for, as is well known, the animals lose about one half their weight during this stage, a loss very largely due to the elimination of intestinal contents and of water. This can be seen in Table II, which shows that the ratio of dry weight to gross weight more than doubles. Whatever may be the significance of the desiccation phenomena revealed by these figures, a unit volume or weight of tadpole tissue cannot be compared directly to one of frog tissue. The figures for the fifth stage and the post-metamorphic animals are, however, comparable.

Table II

Stage 2				Stage 5			
Preparation No.	Gross Weight *grams*	Dry Weight *grams*	Dry Weight / Gross Weight *per cent*	Preparation No.	Gross Weight *grams*	Dry Weight *grams*	Dry Weight / Gross Weight *per cent*
27	1.52	.088	5.8	34	.611	.089	14.6
28	1.84	.120	6.5	25	.710	.093	13.1
29	1.92	.134	7.0	36	.743	.109	14.7
30	1.10	.069	6.3	37	.742	.106	14.3
31	1.48	.094	6.4	38	1.243	.187	15.0
32	1.12	.060	5.3	40	.755	.111	14.7
Average	1.50	.094	6.2 ± .2	Average	.801	.116	14.4 ± .3

Keeping these limitations in mind, we see that the amount of thyroid (cell number or colloid volume) per unit volume of tissue increases but slightly if at all until the hind legs start growing actively. It then shows a remarkable rise, reaching a high point three or four times the original level at the beginning of resorption of the tail (fourth stage). During this stage the behavior of this ratio is problematical, but after metamorphosis it suffers an equally remarkable loss to about one third its previous value.

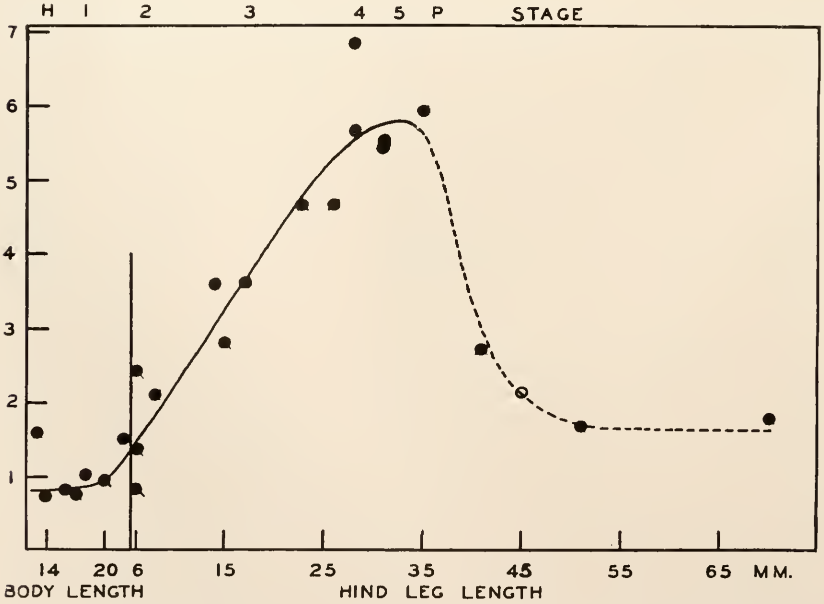

Fig. 1. Ratio of cell count to the cube of the body length plotted against body or hind leg length.

These relationships are clearly shown in the graph, Fig. 1, in which the ratio of cell count to body length cubed is plotted against the body or hind leg length. It is necessary to use body length as abscissa in the first part of the curve, for the hind legs are then but buds, whereas later the hind legs furnish the only adequate measure of the degree of development. This break in the abscissa (shown in the figure by the heavy vertical line) is not of great importance as the ratio remains practically the same until the period of the growth of the hind legs.

Since the thyroid is embryonic in structure before metamorphosis, its functional importance in the tadpole is probably small. After the beginning of metamorphosis, the amount of thyroid per unit of body rises, indicating an increasing importance. As the last stage of metamorphosis, that characterized by resorption phenomena, shows the highest ratio, it seems probable that the thyroid is of special importance in this stage. The subsequent decrease of this ratio in the frog further indicates the importance of the thyroid to the events that take place in the fourth stage.

Further evidence for this conclusion is seen in the fact mentioned above that the epithelium is highest during the fourth stage.

Furthermore, it should be noted that the growth of the colloid is essentially like that of the cell number and at no time is any loss of colloid apparent.

SUMMARY

A quantitative study of the growth of the thyroid gland in relation to metamorphosis reveals the following facts:

1. The number of cells as counted in serial section shows an enormous increase during this process. The major part of the increase occurs between the time the hind legs start active growth and the beginning of the resorption of the tail.
2. The colloid volume behaves in a similar manner, at no time showing a sudden decrease.
3. Calculations of the ratio of cell number (or colloid volume) to the cube of the body length show that the number of cells (or colloid volume) per unit tissue rises but little before the beginning of the active growth of the hind legs. From then until the beginning of tail resorption its value more than triples. After metamorphosis has been completed, this ratio is reduced by about two thirds.
4. The ratio of the cell count to the colloid volume is found to increase four or five times from the half-grown tadpoles to the time of tail resorption. After this it tends to be constant.

These facts are interpreted as indication that the principal importance of the thyroid in metamorphosis is for the latter stages when the tail is being resorbed.

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NEW PHYSIOLOGICAL STUDIES ON CARDIAC MUSCLES OF INVERTEBRATES

IV. The Electrocardiogram of Limulus Polyphemus

M. DUBUISSON

The Marine Biological Laboratory, Woods Hole, Mass.

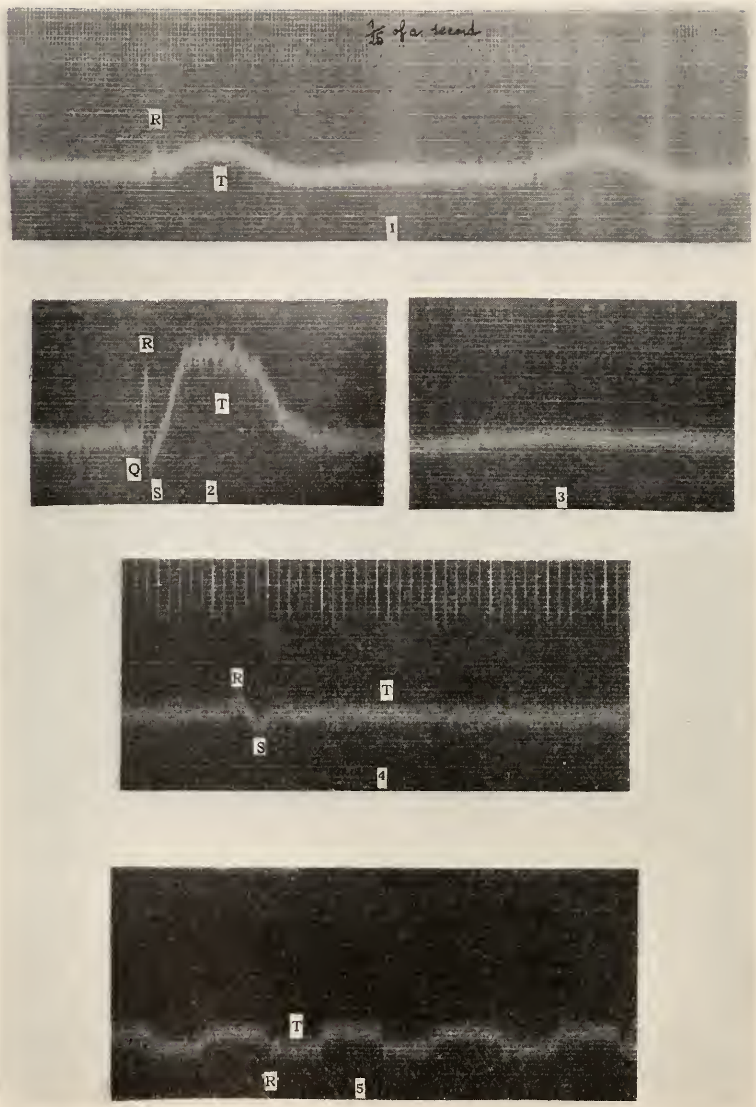
The electrocardiogram of *Limulus* has been studied by Hoffmann (1911, 1922) and Nukada (1918). Both were working on *excised* and empty hearts. The diagrams published by Hoffmann show a mono- or polyphasic action current with very irregular oscillations. According to Hoffmann, the oscillatory character of the electrocardiogram of *Limulus* demonstrated the tetanic nature of the heart beats of this arthropod. According to Nukada, these irregular oscillations are shown by fatigued hearts. The electrocardiograms of fresh animals show only polyphasic variations that may correspond to the *R*, *S* and *T* oscillations of the vertebrate heart. But, according to Hoffmann, (1922) Nukada's diagrams are not demonstrative, as they all show a varying number of oscillations both irregular and complicated.

Edwards (1920) has taken electrocardiograms of *Limulus*, in order to measure the velocity of conduction of the contractions of the *Limulus* heart. He has published these action currents, but without interpreting his results. It is quite obvious that his diagrams are not oscillatory. I have worked in Woods Hole on the action current of the *Limulus* heart, and I have taken special care to vary as much as possible the conditions of the experiments.

A. In the first series of experiments I worked on hearts *in vivo* and *in situ*. In order to expose the whole length of the heart I cut the part of the dorsal tegument which covers that region.

In this condition and if the *Limulus* is firmly attached to a strong and isolated stand, the oscillograms often showed not only the action current from the heart, but also action currents from other muscular motions. But after a while the *Limulus* gets tired, and then it is possible to get electrocardiograms without other disturbances.

The diffuse electrode is composed of a silver rod placed in the dorsal cephalothoracal muscles, for the recording of the electrocardiogram of the whole heart, or placed between the digestive tracts and the heart segment, for the recording of the action current of this region.



Figs. 1–5. In all the records, *A* and *T* are positive, *Q*, *S* and *T′* negative deflexions of the action current.

1. Electrocardiogram from second segment of *Limulus* heart. The active electrode has been sutured on the cardiac muscle. Rhythm: 11 per minute.

2. Ibid from another heart. Subject not motionless. Rhythm: 19. Same velocity as in Fig. 1.

3. Ibid for another heart. The active electrode has not been sutured on the cardiac muscle. Rhythm: 17.

4. Ibid of fourth segment from another heart. Same conditions as in Fig. 3. Rhythm: 17.

5. Ibid from fifth segment from another heart. Subject motionless; the active electrode has been sutured on the cardiac muscle. Rhythm: 17.

The active electrode is composed of a small silver wire which is placed on the cardiac muscle or hooked into this tissue.

My first records gave variable results: some were similar to those obtained by Hoffmann, others to those of Nukada or Edwards. But the comparison of these different curves showed plainly that the irregular oscillatory character of some electrocardiograms resulted: for some, from the interference of skeletal muscle action currents, and for others from the fact that the electrodes, and especially the active silver wire, moved slightly during each contraction.

Figure 2 represents the electrocardiogram of the second segment of the *Limulus* heart. But it shows also, besides the proper electrocardiogram, some accessory oscillations which take place before, during and after the heart contraction, (interference of skeletal muscle action current) and which make difficult the interpretation of the curve of the heart. It is possible to recognize, nevertheless, first a quick and short deflection Q, then two others, R and S, of greater amplitude and longer duration. They are followed by a greater and longer deflection, T, positive as R, and another one, T', in the opposite direction.

Figure 3 represents an electrocardiogram of the same cardiac segment of another *Limulus*. This experiment gives also complicated oscillations, but only during the polyphasic curve, and not before nor after. But in this case the active electrode was not attached to but merely lying on the heart muscle, and certainly was moving slightly during the contraction.

Figure 4 is taken under the same experimental conditions as Fig. 3, but on the fourth segment of a new heart. We can recognize the R, S, T, and T' deflections.

Figure 1 shows an electrocardiogram of the second segment of a heart of a *Limulus*, which did not move during the recording. The active electrode had been sutured on the cardiac muscle by means of a very small thread. We recognize in this diagram the R and T deflections without the other and complicated oscillations.

Figure 5 is taken from the fifth cardiac segment of another *Limulus*, quite motionless. The active electrode had been sutured as previously. We still recognize R and T deflections, without oscillatory character.

These experiments show that if the muscular motions of the animal or the bad fixation of the electrodes do not complicate the cardiac action current, this action current is only composed of a small number of deflections.

A careful study of the electro-cardiogram has given the following relations between the different deflections of the polyphasic action current:

TABLE I

No. and Corresponding Figures	Q wave		R wave		S wave		T wave		T′ wave		Rhythm per Minute	Total Duration of the Waves in σ	Observations
	σ	mv.	σ	mv.	σ	mv.	σ	mv.	σ	mv.			
36 Fig. 2	36	0.08	108	0.50	108	0.28	1800	0.64	840	0.01	19	2052	(1)
51 Fig. 1	—	—	180	0.15	—	—	1740	0.72	600	0.02	11	1920	(2)

(1) Electrocardiogram segment 2 *in vivo.*—Very strong beats.
(2) Electrocardiogram segment 2 *in vivo.*—Strong beats.

The two electrocardiograms studied above correspond to Figs. 1 and 2. These show:

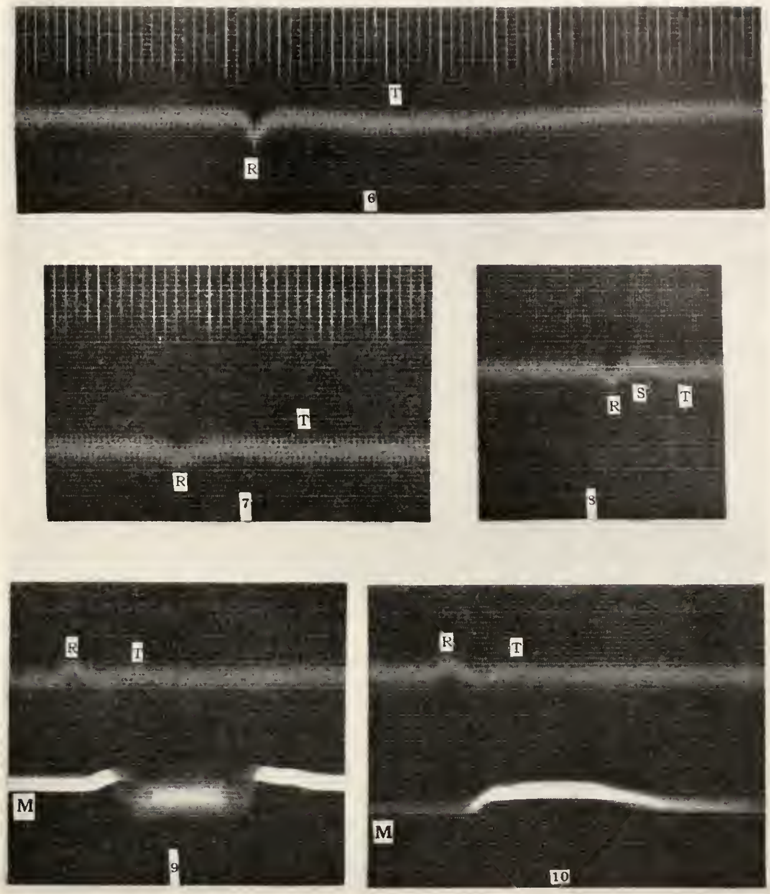
1. For animals with vigorous hearts, three fast deflections Q, R and S, analogous to those of vertebrate heart, a slow deflection T, located on the same side of the base line as R, and following with an opposite deflection T'.
2. Weak hearts show only the R, T and T' deflections.

B. In a second series of experiments we have been working on heart *in vitro.* The heart was fixed on the cork bottom of a basin which contained just enough sea-water to immerse the heart. The electrodes, composed of two silver rods, were vertically fixed in the cork, at a few mms. on the left and right side of the heart, in order to avoid any contact between the two at any moment of the cardiac beat. This disposition gives very good and constant results. The electrograms I obtained were the same for each segment of a definite heart. Figure 6 shows an electrogram of the fourth segment of a heart placed in sea-water. We get two deflections, R and T, without irregular oscillations.

Figure 7 represents the electrogram of the fourth segment of the same heart, after a stay of 40 minutes in sea-water; Fig. 9 after a stay of 140 minutes. In all these electrograms we find R and T and, if we look with a magnifying glass, we may find Q and S, but never any irregular oscillations.

Figures 8 and 10 represent total electrograms of two other hearts that had been immersed for 60 minutes in sea-water.

All our electrograms of normal hearts immersed in sea-water are always free from irregular oscillations. The polyphasic action current is only composed of the deflections R and T, or Q, R, S and T, that we have found already in normal *in situ* electrocardiograms.



FIGS. 6–10. Symbols as in Figs. 1–5.

6. Electrogram of fourth segment of *Limulus* heart beating in sea-water. Electrodes placed in the medium. Rhythm: 18.

7. Ibid, beating in sea-water for 40 minutes. Rhythm: 15.

8. Total electrogram of a heart beating for 60 minutes in sea-water. Electrodes placed in the medium. Rhythm: 4.

9. Ibid from fourth segment of same heart as in Figs. 6 and 7 beating in sea-water for 140 minutes. Rhythm: 4. *M* = myogram.

10. Same as in Fig. 8. *M* = myogram.

The following table shows the comparative variations of these different deflections for a definite heart, after it has been immersed in sea-water for varying lengths of time. These experiments correspond to Figs. 5, 6, 7 and 9.

TABLE II

No. and Corresponding Figures	*Q* wave		*R* wave		*S* wave		*T* wave		*T′* wave		Rhythm per Minute	Total Duration of the Waves *in σ*	Observations
	σ	*mv.*	*σ*	*mv.*	*σ*	*mv.*	*σ*	*mv.*	*σ*	*mv.*			
40 Fig. 5	—	—	132	0.13	—	—	1650	0.07	—	—	17	1782	(1)
40 Fig. 6	—	—	240	0.10	—	—	1560	0.02	—	—	18	1800	(2)
40 Fig. 7	42	0.02	420	0.06	400	0.03	1200	0.02	—	—	5	2142	(3)
40 Fig. 9	120	0.01	450	0.05	750	0.01	780	0.01	—	—	4	2250	(4)

(1) Electrocardiogram segment 4 *in vivo.*—Strong beats.

(2) Electrogram segment 4 of the same heart, beating in sea-water.

(3) Electrogram segment 4 of the same heart, beating in sea-water for 40 minutes.

(4) Electrogram segment 4 of the same heart, beating in sea-water for 140 minutes.

We can see that *R* and *T* are present in all cases; *Q* and *S* appear only after the heart has been in sea-water for at least 40 minutes. When the heart becomes tired, especially in sea-water, the electrograms are modified as follows:

1. The *R* deflection becomes longer (from 132σ to 450σ). When *Q* and *S* are present, they show analogous variations.
2. The *R* deflection has a minor amplitude (from 0.13 millivolts to 0.05 millivolts). When *Q* and *S* are present, they show analogous variations.
3. The *T* deflection is shorter (from 1650σ to 780σ).
4. The *T* deflection has a minor amplitude (from 0.07 to 0.01 milliv.).
5. The total duration of action current becomes greater (from 1782σ to 2250σ).

These observations enable me to conclude that when the heart becomes tired, especially in sea-water, the deflections of the action current show a minor amplitude, while the complex *Q*, *R*, *S* becomes longer and *T* shorter.

C. In some of my experiments I have recorded simultaneous action current and heart contraction of a specific part of the heart. In all these records I always saw:

1. That the T deflection of the electrocardiogram takes place at the same time as the systole of the heart:

2. That the hearts with slow systoles of great amplitude give a longer T deflection, of great amplitude also.

3. That the T deflection has a minor amplitude and a shorter duration when the cardiac systole is faster and shorter.

Thus, the T deflection of the electrocardiogram of Limulus represents simultaneously and proportionally the cardiac muscle activity.

In a great number of electrocardiograms, the T deflection takes place simultaneously with the cardiac systole and ends with it. But in others, the T deflection follows immediately, without rest period, the S deflection, and its amplitude is often so small that it is nearly impossible to locate exactly its beginning or its end.

Summary and Conclusions

The electrocardiogram of *Limulus* has no oscillatory character in normal conditions. Irregular oscillatory deflections are observed when the *Limulus* is not motionless, or when the electrodes are not fixed in such a manner that they could move slightly during the heart beats.

The electrocardiogram of *Limulus polyphemus* is composed of a series of deflections analogous to the ones that we already know for the vertebrate heart. For that reason, we have indicated these different deflections by the same letters: Q, R, S and T. We have called T' an accessory and not always present deflection that follows T. In the action current of the heart of *Limulus*, R and T are always present; Q and S appear only for vigorous hearts or for hearts with a considerably diminished activity.

Fatigue, especially when experiments are carried on in sea-water, modified the aspect of the electrocardiogram in this way: that the complex Q, R, S and T shows a diminution of amplitude, while Q, R, and S are longer and T shorter.

The T deflection is synchronous with the heart systole; in most cases it begins and ends with it. The amplitude and duration of T deflections are proportional to the amplitude and duration of the systole.

The Q, R, and S deflections always take place before the contraction. I think they represent electric discharges in the muscle preparatory to the contraction. These preparatory discharges are smaller in amplitude and slower in time in tired hearts than in fresh preparations.

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METHODS FOR THE CULTIVATION OF CATTLE CILIATES

SYDNEY MARGOLIN

(*From the Department of Zoölogy, Columbia University*)

The problem of cultivating cattle ciliates in artificial media has attracted almost every worker who has concerned himself with either the physiology of ruminant digestion or the general morphology of these exceedingly interesting Protozoa. In general the nature of the problem did not necessarily warrant an empirical approach. The diet and its chemical structure, and the fact that the physiology of cattle was reasonably well known, all pointed to a type of medium easily evolved and maintained.

As a result, the earlier workers, Coste (1864), Edwards (1864), Pouchet (1864), and others, used various kinds of hay infusions, but with no success. The later workers, Fiebiger (1923), Becker and Talbott (1927), taking into account their natural habitat, suggested that the Infusoria might require more complex media containing various proteins and their derivatives. Knoth (1928), however, devised a unique medium that came nearest to success. Into suction flasks containing his hay infusions he introduced a mixed atmosphere of carbon dioxide and methane in proportions of 65 and 35 per cent respectively. The pH was kept as near to 6.8 as possible, and magnesium oxide was used to neutralize the fatty acids produced. Some of his Infusoria lived for 107 hours in a medium thus prepared.

It has been known for some time that the cellulose derived from grains and grasses constitutes a large part of the ruminant's diet. Schuberg (1888), Certes (1889), Eberlein (1895), Leibetanz (1910), Braune (1913), all observed the ingestion of plant fibres by cattle Infusoria. They therefore concluded that these ciliates were capable of digesting cellulose. In fact, Certes (1889) stated that these Protozoa control the cellulose fermentation, and are capable of storing glycogen.

But it is by no means agreed that these Infusoria take part in the cellulose digestion by the host. Biederman (1911), and Scheunert and Schillbach (1927), believe that these parasites are commensals. No worker, however, has proven intracellular digestion within the Infusoria. In discussing the utilization of ingested particles, Becker, Schulze, and Emmerson (1930) wrote, "When this phase of the problem is restudied, appropriate tests should be used to determine definitely that the elements reduced are true cellulose. It is possible that certain plant elements undergo disintegration, while others do not."

A most important and generally agreed on fact is the ability of bacteria to digest cellulose. Omelianski (1902, 1904), described two types of cellulose-splitting bacteria, one of which carries the breakdown to hydrogen and the other to carbon dioxide and methane—all of these gases being present in the rumen. Kellerman and McBeth (1912), claiming that Omelianski's cultures were impure, isolated thirty-six active species, all of which rapidly decomposed cellulose with formation of organic acids but no gases. The acids described, acetic, lactic etc., have been demonstrated in the rumen. Reichenow (1927) discussed the possible importance of bacterial activity in cellulose digestion by ruminants. Woodman and Stewart (1928) isolated a bacillus from manure piles which actively fermented cellulose. But the decomposition products such as the acids and the gases, are of little value to the ruminant. The gases are excreted as waste, and the acids which may be metabolically useful to the bacteria are reduced and excreted in turn.

Therefore in order for cellulose decomposition to be useful to the host, the cellulose molecule must undergo hydrolytic cleavage to the soluble and nutritive dextrose molecule. There are certain cytases capable of effecting this hydrolysis, but none has been demonstrated in the alimentary tracts of higher animals.

According to Pringsheim (1912), bacterial digestion of cellulose gives rise to an intermediate disaccharide, cellobiose, which bears the same relation to cellulose that maltose does to starch. Cellobiose splits into two molecules of glucose when acted upon by a cellobiase. Hence this equation:

Bacterial digestion of cellulose—
Cellulose——Cellobiose (glucose—b—glucoside)——Dextrose.
Enzymes on starch—
Starch——Maltose (glucose—a—glucoside)——Dextrose.

The analogy between the two digestion processes is apparent.

Since no cellobiase has been demonstrated in ruminants, further reduction to dextrose is probably brought about by the bacteria themselves.

It was necessary then to devise media containing cellulose which was undergoing hydrolysis somewhat in accordance with the systems described above. In accordance with this hypothesis, the following media were devised and used successfully:

Agar	2 grams
Peptone	1 gram
Lemco	0.5 gram
Locke's Solution	100 cc.

The whole was boiled until solution resulted, then tubed and sterilized in the autoclave. The pH was adjusted to 6.8 with a solution of sodium bicarbonate by means of colorimetric standards.

On these slants two kinds of fluid covers were used:

(1) 10 cc. of a mixture of equal parts of a dense hay infusion and saline citrate, to which was added a few shreds of pulped Whatman's ashless quantitative filter paper.

(2) 5 cc. of starch solution, and 5 cc. of the following mixture which was devised for Woodman and Stewart (1928) for the cultivation of cellulose-splitting bacteria.

Calcium carbonate	1.20 grams
Sodium phosphate	0.50 gram
Ammonium sulphate	0.25 gram
Potassium chloride	0.10 gram
Tap water	100 cc.

In both (1) and (2) the pH was kept as near 6.8 as possible and magnesium oxide was added to neutralize the acids formed by bacterial action. The bacterial growth was enormous and the pH changed so rapidly that subculturing was necessitated every day. All of the cultures were incubated at 37° C.

The most successful and really amazing growth took place in the following two media:

(3) To a 200 cc. Erlenmeyer flask was added:

Dense hay infusion	25 cc.
Saline citrate	25 cc.
Rice starch	0.5 gram
Magnesium oxide	0.25 gram
Pulped filter paper (Whatman's)	0.5 gram

(4) To a 200 cc. Erlenmeyer flask was added:

Dense hay infusion	100 cc.
Rice starch	0.5 gram
Magnesium oxide	0.25 gram
Pulped filter paper	0.5 gram
Inactivated beef serum	5 cc.

These media were incubated for two days at 37° C. and the pH adjusted to 6.8.

An infusion of bacteria from a successful agar culture number (1), was added. The media were again incubated until it was apparent that the pulped filter paper was attacked by the bacteria. This was evident when after about twenty-four hours the paper became brown. The now ready media were inoculated directly with Protozoa from the contents of the cow's rumen, which in this case contained *Diplodinium*, *Entodinium* and *Isotricha*. After three days the cultures were teeming with the Protozoa.

Medium number (3) proved superior in that subculturing was not required until the fourteenth day, whereas number (4) necessitated subculturing within five days.

On the sixteenth day no moving forms were detected in number (3), but on readjusting the pH to 6.8 (the pH had fallen below six) and adding 25 cc. of saline citrate and 0.5 gram of pulped filter paper, many active Protozoa were observed after five days. This is an exceedingly interesting occurrence and it furnishes the basis of an investigation on the possibility of encystment of cattle ciliates. The four cultures described above were maintained for twenty-four days, after which they were permitted to degenerate.

The success of these cultivation experiments opens the path to a variety of investigations. First, there is the possibility of symbiosis between the ciliates and bacteria, since in these cultures at any rate, the bacteria were necessary in order to hydrolyze the cellulose. Secondly, it throws light on the relation of the Protozoa to the cattle host. These ciliates apparently subsist on some product evolved in the process of cellulose fermentation. Therefore, they are, in a certain sense, food robbers, and thus injurious to their host. Thirdly, a study of the physiology of the cattle ciliates. Fourthly, a study of the life histories of the various ciliate forms present in the rumen, and the influence of artificial media as compared with the natural parasitic habitat.[1]

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BIOLOGICAL ASPECTS OF ULTRASONIC WAVES, A GENERAL SURVEY

E. NEWTON HARVEY

(From the Loomis Laboratory, Tuxedo Park, N. Y., and the Physiological Laboratory, Princeton, N. J.)

Since the first description by Wood and Loomis (1927), of phenomena connected with high frequency, high intensity sound waves, a number of workers have investigated more fully the physical, chemical and biological effects of these supersonic or ultrasonic vibrations. Thus, Harvey and Loomis (1928) described a low power apparatus for direct observation of the effects on cells under the microscope, and Harvey, Harvey and Loomis (1928) continued these observations in more detail. Schmitt, Olson and Johnson (1928) and Schmitt (1929) have described methods of leading the vibrations to an isolated cell by means of a microneedle, together with the changes produced. Harvey and Loomis (1929) have described the destruction of luminous bacteria, and Harvey (1929) the stimulating effect on muscle and nerve tissue. Richards and Loomis (1927), and Richards (1929) have dealt with the chemical effects, as have also Schmitt, Johnson and Olson (1929). Boyle and his co-workers as well as Richards have described a gauge for measuring supersonic intensity. The general methods of producing supersound waves are described in the above papers.

The biological effects of these sound waves may be grouped in five categories:

(1) Whirling of the protoplasm.
(2) Displacement of small particles.
(3) Cytolysis of cells.
(4) Disintegration (emulsification?) of small bodies like chloroplasts.
(5) Stimulation of cells.

Such changes are simply the expression in cells of more general physical and chemical phenomena in liquid media, especially noticeable because of the high intensity and small wave length of ultrasonic waves. They may be classed as follows:

(1) Heating of media which absorb the waves.
(2) Movement of particles into nodes of standing wave patterns, and radiation pressure.

(3) Flocculation (or movement into large aggregates) of particles above a critical size.
(4) Dispersion at liquid-gas, liquid-liquid and liquid-solid interfaces.
(5) Expulsion of gases or vapors from solution (cavitation). This results in a lowering of the boiling point of pure solvents and a discharge of sensitive metastable superheated or supersaturated liquids.
(6) Compression and expansion of media through which the sound waves pass, reaching a maximum in nodes (minimum movement) of standing wave systems and a minimum in internodes (where movement is at a maximum).
(7) Acceleration of chemical reactions.

1. *Heating of Media and Electrical Field*

In regard to the heating of media carrying sound waves, we may distinguish two phenomena: (1) Heating of media due to absorption of sound waves. This effect is not large measured as an average rise in temperature of 10 cc. of water in a test tube, and can be counterbalanced by cooling the fluid with coils carrying cold water. (2) Local heating at interfaces in rapid vibration, where thermal conduction is poor, as when the test tube is held between the fingers. The tube feels quite hot because of the frictional heat developed, but actually the tube rises only slightly in temperature when not vibrating against a frictional surface. The question arises as to whether cells or particles suspended in fluids might not locally rise considerably in temperature due to local friction. Because of the rapid stirring of cells and particles in water and their small size which would facilitate thermal exchange, it does not seem likely that they could rise locally more than one degree above the *average* temperature of the water. Small crystals of ethyl stearate, melting at 30–31° C., do not show signs of surface melting when suspended in water at 28° C. and subjected to supersonic vibration. I feel quite certain that none of the phenomena, especially biological effects, can be connected with *local* heating of the cells, if proper precautions are taken to reduce the *average* temperature of the liquid.

A confusing phenomenon of heating results from the proximity of the media carrying sound waves to the high frequency electrical field. Richards and Loomis (1929) have studied this heating effect, which depends on the frequency and the conductivity of the solution, showing a maximum at the frequencies used to produce supersound waves, around $M/10000$ NaCl. The effect can be completely eliminated by grounded shielding of the medium. The oil about the crystal becomes heated and this heat is conducted to the test tube touched to the oil.

Many control experiments, which need not be restated, have shown without question that no effect of the high frequency electrical field other than the heating effect has any influence in biological phenomena.

2. *Radiation Pressure and Standing Wave Patterns*

The radiation pressure of the sound waves and the appearance of interference patterns and standing wave systems is undoubtedly responsible for the movements and distribution of particles, whether suspended in fluids in test tubes or in living cells.

The radiation pressure is greatest where a wave is reflected from a surface in a standing wave system and is observed in the movement upward of relatively large bodies like *Elodea* leaves in a test tube through which supersound waves are travelling. The whirling movements of particles in cells are due to radiation pressures of different intensity over different regions of the tissue. The explanation of such phenomena depends on a knowledge of the wave pattern, which is often extremely complex, but which presents no unusual peculiarities. The quartz crystal itself does not vibrate uniformly, but its surface shows a complex pattern of moving and stationary regions which may be observed by a modified interferometer, *i.e.*, the amplitude of its movements is of the order of wave-lengths of visible light.

3, 4, 5. *Flocculation, Emulsification and Cavitation*

Flocculation, emulsification and cavitation, especially in relation to the destruction of cells by cytolysis, present some interesting, indeed anomalous peculiarities. It is such effects rather than any chemical action which make supersound waves biologically active.

Johnson has shown that Protozoa (*Stentor, Spirostoma, Blepharisma* and *Paramoecium*) and red blood corpuscles are not destroyed by supersonic radiation, if the dissolved gases are removed by evacuation from the water containing them, or if an increased pressure (of approximately 60 lbs. per sq. in.) is applied to the vessel by connecting it to a compressed oxygen tank. Schmitt and Uhlemeyer (1930) have confirmed this work. Displacement of the dissolved air by a very soluble gas, CO_2, likewise prevents destruction, but blood corpuscles are laked if the air is replaced by H_2 or N_2 at atmospheric pressure. In all cases destruction is associated with the expulsion of dissolved gas (cavitation) from the fluid. If no cavitation occurs, the cells mass in the nodes of standing wave patterns in the tube and are quite unharmed.

I have also been able to confirm these observations in all particulars and in addition to show that the statement applies to other organisms such as *Euglena, Colpidium, Spirogyra, Nitella,* small fish, tadpoles, and *Arbacia* eggs, and also to flocculation of particles (coal, glass, sulphur, marble dust) of a certain critical size and to emulsification of toluol and water, but not to the emulsification of mercury and water.

The flocculation of coal and other particles is easily understood. With gas in solution, cavitated gas bubbles, to which the coal particles stick, are forced by radiation pressure into a clump. Without gas the coal particles are too small for radiation pressure (which depends on the reflection of waves from a surface) to force them together. Too large particles fall so rapidly that flocculation is hard to observe, even if gas is present. Too small particles will not flocculate with gas present because they do not stick to the gas bubbles, and they do not collect in standing waves in absence of gas because Brownian movement counterbalances the radiation pressure against so minute a surface.

If the hydrostatic pressure is increased to 12 lbs., no cavitation occurs and no flocculation of charcoal particles. This shows the importance of cavitation in supplying gas for aggregation of charcoal. On the other hand, if we form small bubbles of hydrogen from amalgamated aluminum in water containing charcoal particles, we can observe aggregation of *charcoal and gas bubbles* at 40 lbs. pressure, *i.e.,* at any pressure which will allow the evolution of hydrogen from the Al surface. Gas bubbles, if present, can be aggregated whether or not they are formed by cavitation.

The flocculation of air bubbles alone can be beautifully shown by the following device. A tube is prepared of the shape shown in Fig. 1, having a rubber stopper carrying a small bacteriological filter in one end. The closed side tube is touched to the oil over the vibrating crystal. After filling with soap solution, by forcing air through the bacteriological filter very minute bubbles rise gradually with quite constant velocity to the surface of the soap. If supersound waves are now passed into the closed end of the side tube, they spread both up and down from the junction with the main tube and instantly force the minute bubbles together into one or more irregular masses. The bubbles do not fuse, since they are surrounded by a soap film, but immediately spring apart when the supersonic radiation is discontinued. The appearance is that of a whitish mass of material bursting into starry bubbles. With water instead of soap solution in the tube, the bubbles fuse completely to form large bubbles, and this effect takes place at the very surface of the filter candle, where much larger bubbles form while the sound waves are passing in than when they are not. Flocculation of air bubbles makes a very sensitive means of detecting supersonics. Mere touching of the side tube to the glass dish of oil containing the vibrating crystal is sufficient to cause flocculation.

The above effects can also be readily observed with other gases. Hydrogen, forming at the surface of Al amalgam in water, is flocculated at the moment of formation by the supersonics. As already stated, increasing the pressure does not prevent the flocculation of hydrogen gas.

Emulsification, which is due to cavitation, and the cytolysis of cells, especially the laking of blood corpuscles, present many characteristics in common. Since the laking of blood corpuscles lends itself to quantitative treatment, most of these studies have been made with a suspension of unwashed turtles' corpuscles containing about 4×10^6 corpuscles per cc. in .65 per cent NaCl in tap water, which will be referred to as blood. A test tube filled with this dilute blood presents the silky appearance due to the optical effect of disk particles and so scatters light that the filaments of an incandescent lamp run at reduced voltage cannot be seen through the blood corpuscle suspension. As laking proceeds and more and more corpuscles are destroyed, the filaments first become visible when about 80 per cent of the corpuscles are destroyed, after 8–20 seconds raying, depending on the conditions of the experiment. Finally all corpuscles are destroyed and microscopical examination shows only nuclei left floating in the fluid. The end point for beginning of appearance of the filaments is very sharp under constant con-

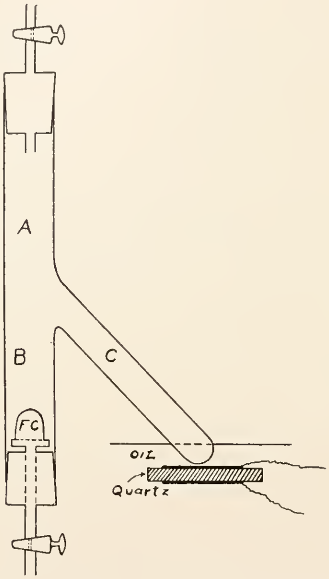

Fig. 1. Tube for studying flocculation of air bubbles.

ditions, and reproducible to within one-half second with similar samples of blood (Jacobs, 1930). The time naturally depends upon the sound density within the tube, a quantity which is extremely difficult to measure, since longitudinal waves are passing up the tube from the bottom and transverse vibration entering from the glass walls. Richards' funnel gauge gives the best measure of the intensity of longitudinal wave systems in a small tube, but does not record the transverse vibrations, which are very strong. In fact, I am not prepared to give absolute values for sound density, nor even relative values and can only say that the radiation is very strong, strong, medium, weak, and very weak, from the settings of a rheostat which controls the filament and plate circuit of the oscillating tubes. The oscillator was the two kilowatt outfit constructed by Wood and Loomis and the quartz crystal a disk 6.5 cm. in diameter and 7 mm. thick with a natural frequency of 338 kilocycles, oscillating in oil. Later experiments were carried out with a ½ kilowatt full wave rectification oscillator at a frequency of 600 kilocycles, the crystal immersed in kerosene. The electrical circuit is illustrated in Fig. 2. This change in frequency is not associated with any change in the phenomena.

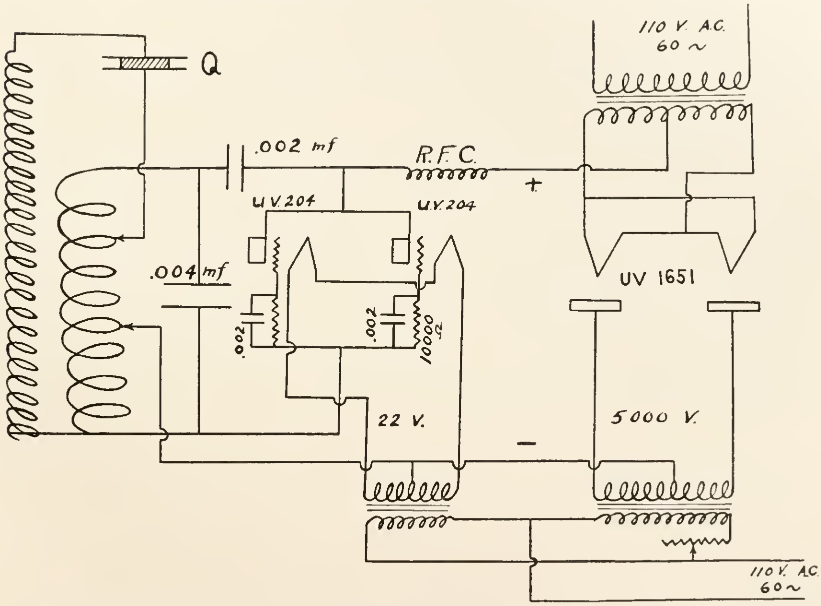

Fig. 2. Electrical circuit of high frequency oscillator for producing supersonic waves.

Perhaps the time for laking may be regarded as a good measure of the sound density, but in the absence of direct comparison by some physical method, one cannot be certain of this criterion. At least time of laking depends, with the same blood concentration and oscillatory field, on the material (whether glass, quartz, thin rubber, dry collodion, or metal), volume and shape of the container, thickness of its walls, nature of the bottom (whether round, flat, a thin diaphragm of metal, etc.), distance from the crystal, and temperature. In the following experiments only one of these factors was varied at a time.

While laking of red blood corpuscles is undoubtedly connected with the cavitation of dissolved gas, there are two points which I believe Johnson did not unequivocally establish. First, the part which *traces* of *cavitated* oxygen might play in chemically producing haemolysis. Second, the protection which might be afforded corpuscles, which always collect in beautiful standing wave patterns in absence of cavitation and in absence of the disturbing movements which accompany cavitation.

Most chemical reactions accelerated by supersound waves are oxidations. Schmitt, Johnson, and Olson (1929) find that iodine is liberated more rapidly from KI in presence of dissolved oxygen by supersound waves and that the effect disappears above about 65 lbs. oxygen pressure, where no visible cavitation occurs. To determine whether corpuscles will lake in *complete* absence of oxygen two types of experiments were carried out.

Blood suspension in Ringer's solution, after two hours' bubbling of hydrogen, previously passed over red-hot platinized asbestos in a quartz tube and led to the experimental tube through lead tubing, laked rapidly with supersonic raying, due to the dissolved H_2. No trace of oxygen was present in this hydrogen. Secondly, blood to which a small amount of $Na_2S_2O_4$ had been added to absorb the oxygen, colorless reduced indigodisulphonate acting as an indicator for oxygen, laked rapidly in supersonics due to the nitrogen it contained. Certainly traces of oxygen can play no part in the laking of blood.

In regard to the second point, the following may be stated. Both reduction of pressure by evacuation and consequent removal of gas, and also increased pressure prevent cavitation. If blood is partially evacuated and rayed under the reduced pressure, some cavitation of gas *under reduced pressure* occurs, but commotion within the tube is not great. However, the movement of this gas breaks up the standing waves, but the corpuscles are not destroyed. If atmospheric pressure is returned over the blood no cavitation is apparent, beautiful standing waves form, and of course the corpuscles also remain unharmed. Cavitation of gas under reduced pressure does not harm the corpuscles.

One might suppose that laking failed to occur because the corpuscles were not *violently* moved by cavitated gas bubbles in and out of the nodes.[1] Accordingly, a small motor stirrer was mounted in a vacuum tight brass chamber having a glass tube sealed to it with de Khotinsky cement in such a way that blood, placed in the test tube, could be evacuated and stirred very vigorously while evacuated. The experiment showed that whereas blood containing air laked in 20 seconds, blood without air (evacuated to 71 cm. negative pressure) did not lake in 6 minutes even though rapidly stirred the whole time that sound waves were passing through it. The stirrer pulls down rarified air bubbles from the surface of the blood, that are flocculated by the supersonics. It is a question, however, whether this stirring produces an agitation comparable with that accompanying marked cavitation.

The stirring and break-up of standing waves can also be effected by rapidly changing the tuning by rotating the variable condenser, but this method of rather gentle electrical stirring does not cause laking of the corpuscles, provided dissolved air is absent. It is, therefore, not the collection in nodes that prevents laking in absence of dissolved gas.

A similar fact can be demonstrated using the tube of Fig. 1 and in addition the fact that *already formed air bubbles together with sound waves have no destructive effects.* The tube is filled with blood and a rubber stopper with a stopcock placed in the upper open end (lower stopcock closed) so that the whole can be freed of air by evacuation. If sound waves are now passed up the side arm, beautiful standing wave patterns appear and no laking occurs in 3 minutes, although a similar amount of blood containing air lakes in 50 seconds. Atmospheric pressure is now returned above the blood and fine bubbles of air caused to rise through *A* and *B* by opening the lower stopcock, disturbing the standing waves in *A* and *B*, but which are still apparent in *C*. However, no laking occurs until 2¾ minutes, when (presumably) enough air has been dissolved to permit laking in *A* and *B*, but no laking occurs in *C*, despite the fact that sound waves entering *A* and *B* must first pass through *C*. The standing wave system remains intact in *C*.

As it can be shown that blood corpuscles do not lake when a suspension saturated or supersaturated with air is suddenly evacuated, resulting in all stages of cavitation of bubbles without sound waves, and it is also true that bubbles plus sound waves do not lake provided very little gas is dissolved, I conclude with Johnson that laking is connected with the initial separation of the gas phase in the liquid by the supersonics.[2] The cause of laking is shifted to the cause of cavitation.

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[1] It should be recalled that in any standing wave system, the greatest movement of particles will be in the internodes, while the greatest changes in pressure (expansion and contraction) will be at the nodes.

[2] Perhaps we should ask whether laking would occur if blood were evacuated three million times in succession, *i.e.*, a number of times comparable to the supersonic frequency multiplied by the seconds to cause laking. A single evacuation may lake a few corpuscles.

Since the cavitation will occur in pure water whose average temperature is continually falling (from absorption of heat by a cooling coil placed in the tube), the separation of dissolved gas must be connected with the decrease in pressure during rarefactions. Why does not, then, the cavitated gas immediately redissolve during the following increase in pressure during condensation? I believe it is because the radiation pressure instantly flocculates minute bubbles to a larger bubble whose surface volume ratio is less and consequently resolution must take place less rapidly. The end result is the cavitation of a few visible bubbles and never the formation of a milkiness due to many minute bubbles. Even with strong beam illumination from the side, one never notices a marked Tyndall effect in water which might be due to small air bubbles.

In capillary active substances like soap, Na glycocholate and Na taurocholate solution there is no visible cavitation, in saponin some cavitation. It is likely that these substances prevent *visible* cavitation by rendering impossible the fusion of minute bubbles formed in the first stages of cavitation. Unfortunately, soap sol. etc. gives a good Tyndall beam even in absence of supersonic radiation, but it looks as if the radiation had liberated minute bubbles that do not coalesce, but redissolve in the following compression. Capryl alcohol added to water does not prevent visible cavitation.

From the biological point of view, the really important question is how cavitation destroys the cell. The simplest assumption is that gas bubbles cavitated *within* the cell burst the cell wall. Gas exchange across a cell boundary is so rapid that we cannot hope to test this view by any attempt to remove gas from the medium around the cells, leaving it within the cell. I did attempt the experiment of removing gas from the fluid around *Spirogyra* or *Euglena* cells, which contain chlorophyll, and then raying them in sunlight, but the cells were not destroyed. Photosynthetic oxygen which should form *within* the cells was apparently not produced in sufficient quantity to destroy by cavitation, so the results were negative and mean nothing.

The cavitation of gas bubbles *within Spirogyra* cells does occur after the cell contents are so broken up as to form a green emulsion and the turgor lost, an indication that the plasma membrane has been destroyed. No gas has been observed in cells with slight displacement of the chlorophyll bands.

Indeed it is unlikely that cavitation would readily occur in minute capillary spaces except with very strong sound waves. Cavitation does occur and blood corpuscles are laked in capillary glass tubes drawn out from a larger test tube whose closed end is touched to the oil above the vibrating crystal. Such a tube is a collector and concentrator of sound energy in the capillary. If the tube is placed in the oil away from the crystal so that the sound density is weaker, standing waves are formed and no laking occurs and no cavitation even though gas is present. On the other hand, blood containing dissolved gas in capillary tubes closed at one end and directly touched to the oil above the vibrating crystal does not cavitate or lake, but standing waves are apparent, whereas in a *larger* tube cavitation and laking occur. As we cannot compare the sound density in the two tubes, the observation may merely mean that the ultrasonic intensity is too small for laking in the capillary tube.

The question of laking in capillary spaces was approached in another way. A small amount of blood (½ cc.) was shaken with a large volume (10 cc.) of N-butyl-phthalate, a fluid slightly more dense and somewhat more viscous than water but harmless for blood corpuscles. When the two fluids were shaken, an emulsion of *blood in N-butyl-phthalate* was formed which showed on microscopic examination a few blood corpuscles lying in the small droplets of salt solution. Such an emulsion rayed 30 seconds showed most of the corpuscles unharmed, whereas the corpuscles in 10 cc. blood shaken with ½ cc. N-butyl-phthalate, forming an emulsion of *phthalate in blood,* lake in 10 seconds. The corpuscles in the capillary globules are protected, but we do not know the sound density in the capillary space.

However, as the boiling point of fluids is raised in capillary spaces and the cavitation of gas in capillary spaces is less readily accomplished by evacuation, we may argue that cavitation is less likely to occur within a cell, and on the whole it is unlikely that destruction of cells is due to bursting by gas liberated within. If cells have a high viscosity, it is also more unlikely that cavitation within will occur readily. Blood containing 5 per cent gelatin and air at atmospheric pressure does not lake nor does the gas cavitate, although much gas is present which can be removed by evacuation. On the other hand, thick egg albumen does not prevent laking under the same circumstances. Dilution of the gelatin with salt solution allows the laking to proceed as readily as in salt solution alone. It must be admitted that evidence of this sort is equivocal in the absence of knowledge as to the sound density.

On the other hand, histological examination of muscle tissue of small tadpoles (Chambers and Harvey) subjected to supersonic radiation and immediately fixed, has disclosed minute spaces under high magnification which look as if they had been left by gas bubbles. They are never present in the unrayed control tadpoles. The muscle tissue also shows evidence of breakdown into globular masses and the number of blood corpuscles is less. We have come to the conclusion that death of small fishes is due to destruction of gill membranes and blood corpuscles. This histological evidence in tadpoles is about the only evidence that minute bubbles might form within cells.

It is well known that a rapidly revolving (2000–3000 R.P.M.) spindle with vanes will break up a suspension of cells and sand (Rowland, 1901). Bacteria are destroyed in this way in about five hours. Thinking that the *minute* gas bubbles might vibrate back and forth, hitting blood corpuscles and rupturing them, I have tried adding all sorts of powders and emulsions to the blood, removing the dissolved gas, and exposing to supersonics, but with negative results. Silica powder, levigated alumina, sulphur, parrafin oil, milk, N-butyl-phthalate and starch grains do not cause laking in absence of gas. However, conditions are not quite the same as when cavitation occurs, especially the explosive violence of the movements in the tube.

The behavior of plant cells like *Spirogyra, Nitella* and *Elodea,* in supersonic radiation, is of special interest, since the protoplasm is under a turgor pressure of 4–5 atmospheres and the outer surface of the protoplasm protected by a cellulose membrane, strong enough to withstand the turgor pressure. Nevertheless, all three cells are thoroughly broken up and disintegrated with loss of turgor by the sound waves. In *Spirogyra* there is the break-up of the chlorophyll spirals with loss of turgor, the chlorophyll becoming diffuse in many cells; in *Nitella* the layer of chloroplasts contiguous to the cell-wall is violently projected into the cell sap in regions where the sound density is high; in *Elodea* there is complete emulsification of the cell contents and breaking of the walls so that many cells become empty. With a gas tension of one atmosphere in the water and 100 lbs. per square inch hydrostatic pressure on the water containing *Elodea, Spirogyra* and *Nitella,* destruction does not occur even when very strong supersonics are passed through the fluid. As will be shown later for blood corpuscles, the effect of pressure in preventing destruction of cells by sound waves depends largely on the tension of the dissolved gas. If the gas in solution is in equilibrium with the pressure, then the *Elodea, Spirogyra* and *Nitella* are rapidly destroyed at 100 lbs. pressure.

Removal of gas by evacuation does not prevent the tearing of *Elodea* cells by supersonics, although *Spirogyra* and *Nitella* are not so readily affected and may not be harmed at all if the supersonic intensity is low; whereas there is complete disintegration if dissolved air is present, at the same low sound density.

The anomaly presented is this. How can gas be cavitated within a cell under 4–5 atmospheres (60–75 lbs. per sq. in.) turgor pressure when all experiments indicate that 15–65 lbs. hydrostatic pressure, depending on the tension of gas and supersonic intensity, is sufficient to prevent cavitation of gas from water solution? We cannot believe that the gas dissolved *within* plant cells is under a higher tension than one atmosphere even though the turgor pressure is greater. On the other hand, how can externally cavitated gas destroy a cell surrounded by so tough a cellulose wall? Unless, perhaps, it is the violent knocking on the cellulose wall by cavitated gas bubbles which finally breaks down the cell permeability and abolishes the turgor pressure within, when cavitation inside may complete the thorough break-up of the cell. Schmitt and Uhlemeyer (1930) find that *Spirogyra* subjected to ultrasonics too weak to rupture the cell wall or cause visible changes, are less resistant to penetration of NaOH.

From a study of plant cells one is almost led to the conclusion that it is the rapid movements accompanying the cavitation rather than the cavitation itself which breaks them up. I think the plant cell experiments negative the view that destruction is due to expansion of cavitated gas within, bursting the cell-wall.

Attempts were made to analyze the break-up of cells by means of moving pictures with a high speed f 1.8 camera taking 128 pictures a second. The exposure is only 1/200 second, but perfectly timed negatives can be obtained with a No. 3 objective (10 ×) and No. 2 (6 ×) or No. 4 (10 ×) ocular if a beam from a Pointolite lamp is used for illumination. *Spirogyra, Nitella,* and *Arbacia* eggs proved to be favorable material. They were mounted under a cover glass directly on the vibrating quartz crystal, using a low power (75 watt) oscillator. The results indicate that particles or previously present air bubbles caught under the cover glass move with extraordinary rapidity, such that they may appear in one frame and disappear in the next. An *Arbacia* egg may be completely cytolysed in one frame (1/128 second), so that there is no difficulty in telling (by noting the movement of small particles) when the supersonics have been turned on. The process of cytolysis is too rapid to be analysed at this speed (128 pictures per second).

On the other hand, the twistings of *Spirogyra* spirals and the tearing of *Nitella* chloroplasts from the wall is much slower, as is also the forcing of organisms like *Euglena* into standing wave patterns. Effects within *Nitella* and *Spirogyra* filaments can be just recognized in 1 frame (1/128 second), are marked in 4 to 5 frames (1/25 second) and very marked in 12 frames (1/10 second) from the time the supersonics are turned on. The pictures do not disclose air bubbles within the cells, nor do they give a clue to the cause of destruction. The filaments are held in place by the cover glass and do not move during the supersonic raying.

The question arises as to whether stimulation is also due to cavitation. Stimulation of tissues does not easily occur with supersonics except under special conditions. A frog's sciatic-gastrocnemius preparation immersed in Ringer's will give submaximal twitchings, and a turtle's heart under similar conditions is stimulated to extra beats when supersound waves pass through the fluid, but no stimulation results on merely touching tissues to solids carrying sound waves.

Will evacuation of gases from the Ringer's solution prevent this stimulation? To determine this, an isolated turtle's ventricle was mounted in a rubber-stoppered test tube carrying a stopcock (for evacuation), a support, and a short spring lever, in such a way that contraction of the ventricular muscle could be observed by movement of a beam of light reflected from a small mirror on the lever. Several experiments using different ventricles all showed that it was much easier to obtain contractions when gas was dissolved, whether air or oxygen-free hydrogen (since it was thought that lack of oxygen on evacuation might lower the irritability of the ventricular muscle), than when the gas was removed by evacuation. There was, however, frequently some contraction in absence of gas. The gastrocnemius-sciatic preparation is also much easier to stimulate when air or hydrogen gas is dissolved than after evacuation.

Since pressure plays so important a part in the destruction of cells by supersonics, it seemed necessary to determine the relation between the pressure on the fluid and time of laking of its contained cells. Dilute blood was used and the time was determined by noting the appearance of incandescent lamp filaments viewed through the suspended blood corpuscles, as described on page 310, taking care to keep all conditions (which might affect the time) constant in any one series of pressure experiments. Preliminary work showed that the more concentrated the corpuscle suspension, the greater the laking time, but there is not a direct proportionality. Consequently time cannot be compared in series of experiments run with different samples of blood, but the form of the curves is similar.

Preliminary work also showed that in general, the greater the supersonic intensity, the shorter the time of laking. As previously explained, the actual supersonic intensity cannot be stated, but relative intensities only can be given in terms of the steps of a rheostat in the plate transformer circuit of the oscillator controlling the voltage across the crystal. The times for laking were:

Step 31, all resistance in, weak supersonics	11	seconds
Step 25, some resistance out, medium supersonics	8	"
Step 17, more resistance out, strong supersonics	5.5	"
Step 13, more resistance out, very strong supersonics	8	"
Step 9, more resistance out, very strong supersonics	8	"
Step 6, most resistance out, strongest supersonics	4.5	"

Why steps 13 and 9 with less resistance and greater voltage across the vibrating quartz crystal should be less efficient in laking the corpuscles than step 17 cannot be stated. This is one of the unexplained anomalies which have appeared in this work.

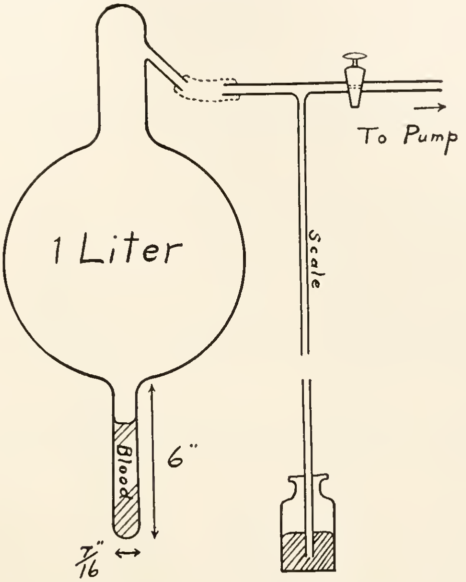

Fig. 3. Vessel for studying laking under diminished pressure.

The actual pressure experiments can be divided into those in which the pressure is increased above atmospheric and those in which the pressure is decreased by evacuation. For the latter the glass vessel illustrated in Fig. 3 was used, a 1 L pyrex distilling flask with the neck sealed off and a pyrex test tube sealed in the bottom. The blood corpuscle suspension is placed in the flask, which is then connected to a vacuum pump, evacuated to a certain pressure and the blood thoroughly shaken with air at this pressure to bring it into equilibrium. The test tube is then touched to the oil above the vibrating crystal and the time of laking noted. The graph shown in Fig. 4 gives a series of runs under the same conditions with three different intensities of supersonics. Time in seconds for laking is plotted as ordinates and pressure in mm.

of mercury as abscissæ, after deducting 23 mm. for pressure of water vapor at 24° C.

It will be observed, first, that there is a rather well-defined pressure at which the time of laking becomes greater, and at slightly lower pressure this time becomes practically infinite, even though there is still

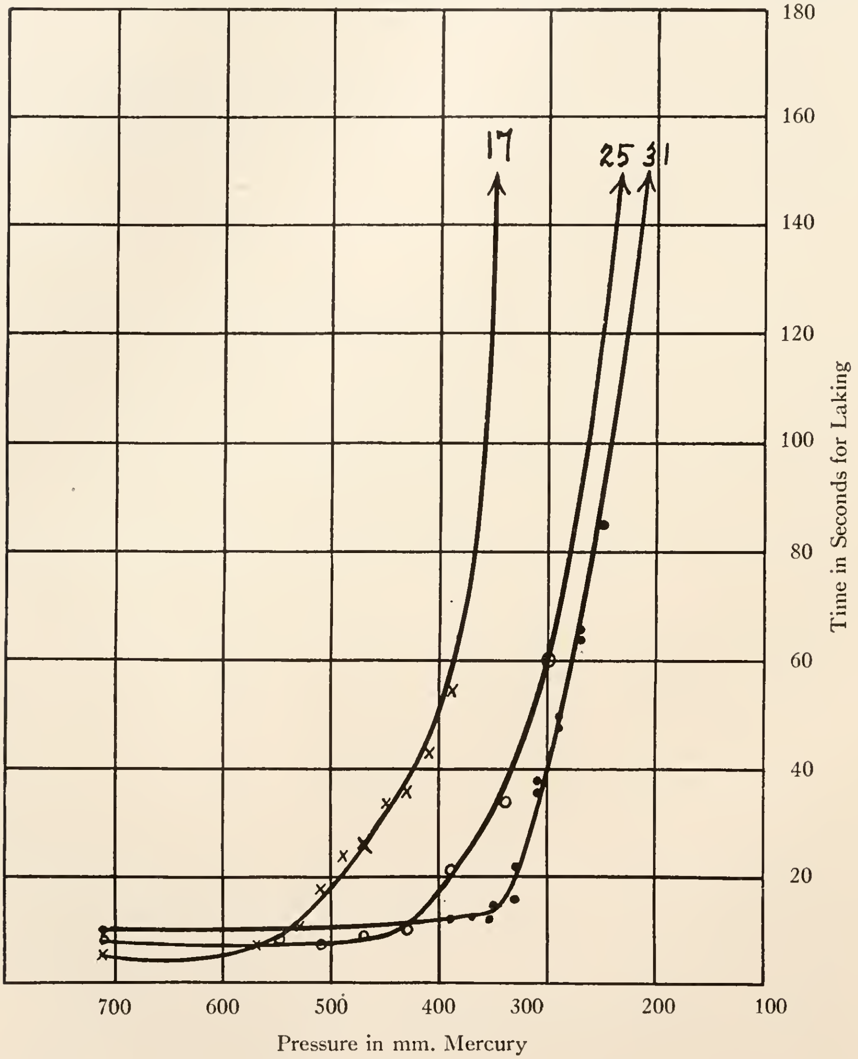

FIG. 4. Graph of time in seconds for laking of turtle's blood plotted against pressure in mm. Hg, the dissolved gas in equilibrium with the pressure, with three different intensities of supersonics, strong 17, medium 25, and weak 31.

air dissolved which cavitates under the reduced pressure. In four different runs at different times this critical pressure with 31 supersonics was about 350, 380, 430, and 460 mm. Hg. Second, with more intense supersonics (rheostat on steps 25 and 17 as compared with 31) *a greater amount of gas may be dissolved without laking.* This is an extraordinarily anomalous fact for which I have no explanation. The

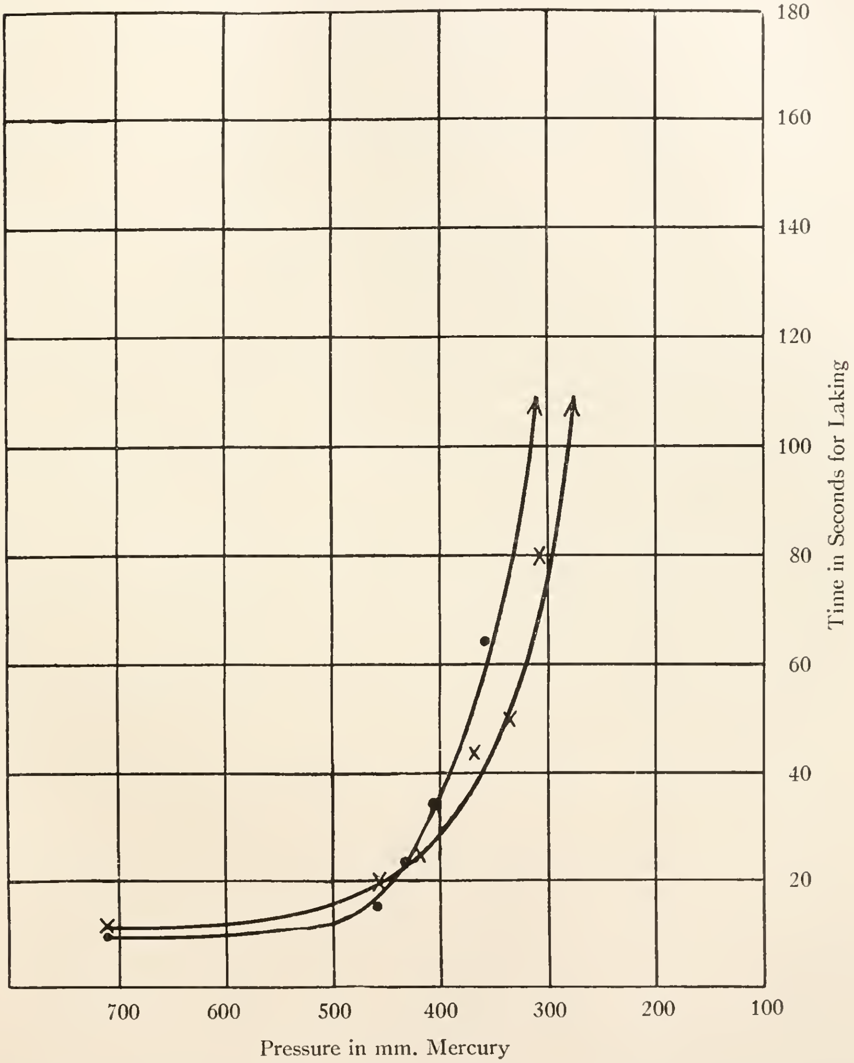

FIG. 5. Graph of time in seconds for laking of turtle's blood plotted against pressure in mm. Hg, the dissolved gas, oxygen (x) and air (●) in equilibrium with the pressure, weak (31) supersonics.

expectation would have been that greater supersonic intensity would require the removal of more dissolved air to prevent laking. The same result has appeared in three entirely separate sets of experiments.

The next experiment was designed to determine the influence of the amount of gas and kind of gas dissolved on the form of the curve. Since 1 cc. water dissolves .03102 cc. of oxygen and .01870 cc. air measured at 0° C. and 760 mm. from 760 mm. of gas, there will be about 1.72 times as much oxygen in solution as air. The observation vessel was arranged so that blood could first be thoroughly evacuated to remove gas and then connected with oxygen or air, respectively, and shaken at various pressures to bring it into equilibrium, and the time for laking by weak supersonics of 31 intensity noted. The results are shown in the graph of Fig. 5. The curves for air and for oxygen are practically identical. If anything, it is necessary to have a little lower pressure to prevent laking with oxygen dissolved as compared with air, a result which might be expected.

It can be shown very definitely that if much more gas is dissolved in blood suspension than corresponds to equilibrium with the pressure of gas over the liquid, laking occurs, whereas it would not occur under equilibrium conditions. For example, blood with air in equilibrium at atmospheric pressure is placed in the observation tube and the pressure over the blood reduced to 340 mm. Hg *without shaking.* Laking occurs in a few seconds, whereas no laking would occur in 3 minutes' exposure if the blood contained gas in equilibrium at 340 mm. Hg.

The question of gas equilibrium becomes extremely important when pressures above atmospheric are studied. For this purpose two types of observation chambers have been used, the gas studied being air. In one, for increasing pressure without dissolving more gas, a pyrex test-tube is sealed by deKhotinsky cement to a brass chamber connected with a pressure gage and bicycle tire valve (Fig. 6*B*). The pyrex tube pressure (Fig. 6*A*). The chamber can be completely filled with blood saturated with air at 760 mm. Hg, the petcock closed and the pressure increased by turning the thumbscrew. It is found that blood corpuscles under 12 lbs. pressure or greater, with weak (rheostat 31) supersonics, do not lake, but laking occurs in a few seconds if the pressure falls below 11 lbs. For stronger supersonics (rheostat 17) there is laking below 13 lbs. but none above 15 lbs. per sq. inch. Between these critical pressures cavitation and laking may or may not occur, *i.e.,* there appears to be a very critical condition for cavitation such that cavitation occurs like a little explosion in the liquid, followed by a period of rest and a moment later by another explosion and so on. These explosions, noted by Johnson with low sound densities, cause great commotion and movement in the liquid, breaking up the standing wave patterns. It is very likely that the corpuscles are subjected to tearing forces during these explosions which could only be accomplished by rotating vanes after many hours exposure.

The second type of pressure chamber, for obtaining equilibrium between gas and liquid, is a pyrex tube sealed to a brass tube connected with a pressure gage and bicycle tire valve (Fig. 6*B*). The pyrex tube is only partially filled with blood, the chamber pumped to the desired pressure with a bicycle pump, when thorough shaking of compressed air and blood brings the system into equilibrium.

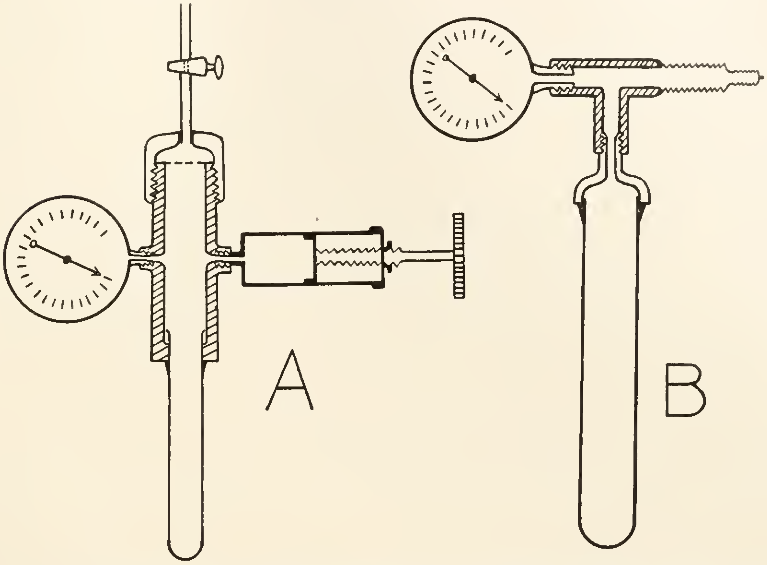

Fig. 6. Vessels for studying laking and increased pressure. *A*, for gas dissolved at atmospheric pressure; *B* for gas in equilibrium with the pressure.

Using this chamber, it is found that cavitation and laking cannot be prevented even up to 100 lbs. per square inch, if the gas is in equilibrium. The time for laking becomes longer, perhaps 20 seconds at 100 lbs. as compared with 5 seconds at atmospheric pressure. I believe the 60 lbs. pressure found by Johnson to prevent destruction of Protozoa is the result of the fact that under his conditions the fluid was only partially in equilibrium with the gas under increased pressure.

For a given sound density it would appear that the tension of gas in solution in relation to pressure was the most important factor in determining cavitation of a fluid and consequent destruction of cells suspended in it. With a lower gas tension than corresponds to equilibrium with the gas phase, increasing the hydrostatic pressure will prevent cavitation and laking; the higher the gas tension, the more the hydrostatic pressure must be increased. At equilibrium, increasing the hydrostatic pressure (to 100 lbs.) will not prevent cavitation. Pressures above this have not been studied. Whether the ratio of hydrostatic pressure to gas tension is a constant and bears a relation to the negative pressure developed during the rarefaction phase of a sound wave cannot be stated at the present time. Methods of actually measuring the pressure changes during passage of high intensity sound waves must be developed. Boyle, Taylor and Froman (1929) believe cavitation is not due to disruption of the liquid with bubble formation *de novo,* but is due to radiation pressure forcing small invisible bubbles together, since their measurement of the energy intensity in a supersonic beam is only one-tenth of that required by a simple theory of disruption by harmonic pressure. They argue that if bubbles must be actually generated, time should be a factor in their formation and it is highly improbable that a bubble could form in 10^{-6} seconds, for it is easily demonstrated that sound waves of one million frequency will cause cavitation of dissolved gas.

The study of cavitation by various means offers an extremely interesting and rather neglected field for work, but as it lies in the realm of pure physics rather than biology I do not propose to follow it up even though the laking of blood corpuscles by cavitation offers excellent quantitative material for its study. In regard to the mechanism of cell destruction by cavitation, I believe it is due rather to the rapid striking of cells by minute cavitated air bubbles and the general violent commotion accompanying cavitation than to the expansive bursting of the cell by gases cavitated within. The chief evidence for this view is the behavior of plant cells described on page 316.

In conclusion I wish to thank Mr. Alfred L. Loomis for the generous hospitality of his laboratory, where practically all of the experiments were performed, and for his great interest and valuable suggestions during the progress of the work. I also express my deep obligation to Dr. William Richards of Princeton University for information during our discussions of the experiments, much of which is embodied herein, and to Mr. Charles Butt, my research assistant, for the loan of his high frequency oscillator.

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A COMPARATIVE STUDY OF DAILY WATER-INTAKE AMONG CERTAIN TAXONOMIC AND GEOGRAPHIC GROUPS WITHIN THE GENUS PEROMYSCUS

LLEWELLYN G. ROSS

(*From the Carnegie Institution of Washington and the Scripps Institution of Oceanography of the University of California, La Jolla, California*)

Introduction

It is a widely accepted generalization that many groups of mammals exhibit differences in water consumption which are conditioned upon the available water supply in their respective environments. Bailey (1923) brings out this fact in a striking manner. He states that certain forms exist in hot, barren areas where there is no open water, no available subterranean water, and no precipitation for periods of months or even years. These animals are present in large numbers and are "found in perfect health and good bodily condition with abundance of internal fluids and secretions." Bailey contrasts this type with such animals as the Eastern gray squirrels which "require water once or twice a day and drink a considerable amount at a time." The same author (1923) describes the food habits of several xerophilous forms, stating that their water supply is obtained from seeds, roots, succulent plants, etc. Babcock (1912) shows that the carbohydrates, proteins and fats of foodstuffs are converted into water which is sufficient for a large share of an animal's vital activities.

Whatever may be the actual source of water supply for xerophilous mammals, the fact remains that they are capable of adjusting their vital economy to a very restricted supply of free water.

Dice (1922), in an effort to explain the different habitat preferences of two species of *Peromyscus*, *P. maniculatus bairdii* and *P. leucopus noveboracensis*, tested their water intake in the laboratory. Neither of these forms is xerophilous. Both are found in Illinois, *P. m. bairdii* being a prairie form while *P. l. noveboracensis* is a forest dweller. Dice found no significant difference in water intake between the two species and concluded that "different water requirements cannot be the factor causing the different habitat limitations" of the forms tested.

While acting as research assistant to Dr. F. B. Sumner during the year 1929–30, under a grant from the Carnegie Institution of Washington, the writer was given the opportunity to investigate further the question of differences in water consumption. The experimental subjects used were representatives of five taxonomic groups of mice within the genus *Peromyscus*. These groups were:

P. maniculatus sonoriensis,
P. maniculatus gambelii,
P. maniculatus rubidus,
P. eremicus eremicus,
P. eremicus fraterculus.

The species *maniculatus* is distributed widely over the North American continent. So ubiquitous is this group that Osgood (1909, p. 17) states that "it is probable that a line, or several lines, could be drawn from Labrador to Alaska, thence to Southern Mexico throughout which not a single square mile is not inhabited by some form of this species." The three subspecies of *maniculatus* with which we are here concerned are distributed as follows. *P. m. sonoriensis* is found in arid and desert regions, chiefly in the southwestern United States. *P. m. gambelii* is found along the California coast, south of San Francisco Bay, in the Great Valley of California and also in central Oregon and Washington, east of the Cascades. *P. m. rubidus* confines itself to the humid coastal region from San Francisco Bay, north to the Columbia River.

P. eremicus, which is placed in a distinct subgenus from *maniculatus*, is much more restricted in its geographic distribution. The various subspecies of *P. eremicus* are confined to arid and semi-arid portions of the southwestern United States and adjoining parts of Northern Mexico (Osgood, 1909, pp. 239–240). *P. e. eremicus* is found exclusively in distinctly arid localities, while *P. e. fraterculus* confines itself to the semi-arid coastal region of California, south of Los Angeles, and of northwestern lower California.

It might be expected, on the basis of their varying climatic environments, that these five subspecies would display interesting differences in water intake. The large stock of *Peromyscus* maintained by Dr. Sumner at the Scripps Institution at La Jolla offered ample material for the present investigation. All five subspecific groups described above were represented by "C_1" stock of known age and parentage.[1] It was a comparatively simple task to measure the individual daily water consumption of a portion of this stock. The details of this procedure will be described later.

The problem to be investigated may be briefly stated as follows:

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[1] "C_1" is a term used by Dr. Sumner to designate the first generation born in captivity.

are there significant differences in water intake among the five racial groups of *Peromyscus* discussed above? If so, are the differences specific (between species) or subspecific (among diverse races within a species), and how are these variations related to the environmental and phylogenetic backgrounds of the groups in question?

MATERIALS AND METHODS

Each animal was kept in an individual stock cage, 16 × $9\frac{1}{2}$ × $9\frac{1}{2}$ inches in dimensions, which was divided into a nest compartment and a food compartment. These two compartments were of equal size and were intercommunicating, the latter having a screen front, the former being closed.

In the food compartment the water-supply contrivance was installed. This apparatus had been devised by Dr. Sumner, previous to the time at which the present investigation was undertaken. It was essentially an inverted rimless test tube, resting in a shallow round aluminum dish, $1\frac{1}{4}$ inches in diameter and 3/16 of an inch deep. The test tube was of 16 cc. capacity, and was graduated in cubic centimeters. A small notch in the edge of the tube allowed air to enter this and water to escape into the dish, as it was depleted by the animal's consumption and by evaporation. The device was set up for use as follows. After the tube was filled, the top was covered by the aluminum dish, and it was then quickly inverted and attached firmly by a clamp, in such a manner that the edge of the tube just cleared the surface of the aluminum dish. The water level was then brought down to the 0-cc. graduation by removing small quantities with a pipette.

Distilled water was used. In order to rule out variations in water-intake referable to changes in kind of food, the modified McCollum diet used by Slonaker (1925) was employed. This consisted of the following proportions by weight.

Ground whole wheat	3375
Whole milk powder	500
Casein	750
Sodium chloride	50
Calcium carbonate	75
Sifted ground alfalfa	150
Unsalted butter	250

No green food was given. It may be noted here that under Dr. Sumner's method of caring for the mice they are given no free water. The animals' moisture requirements are met by giving cactus and lettuce, as well as a mixture of alfalfa meal and wheat germ, moistened with milk and cod-liver oil. In a state of nature it seems likely that

Peromyscus, throughout much of its range, does not have access to free water. Therefore it appears that satisfying moisture requirements by drinking free water was a unique experience for the animals tested.

During the entire period of the experiments, the temperature in the experimental room varied from 21° to 24° C., the mean lying between 22° C. and 23° C. Relative humidity was much more variable, the weekly means varied from 40 per cent to 83 per cent. The mean for the entire period was 62 per cent. The maximum variation in humidity during any one experiment was from 30 per cent to 70 per cent, with the mean about 50 per cent. This range during an experiment was exceptional, the normal range being through about 20 degrees. It is obvious that the only strictly fair comparisons are between animals which were tested simultaneously.

The daily water intake of each individual was recorded for a period varying from two to three weeks. During the experimental period, each mouse was transferred from one cage to another in rotation every second day. This procedure was intended to eliminate variations due to the set-up of the water-supply devices.

Four different racial comparisons were made as follows:

(*a*) between *P. m. sonoriensis* and *P. e. eremicus*, involving 55 individuals.

(*b*) between *P. m. gambelii* and *P. e. fraterculus*, involving 57 individuals.

(*c*) between *P. m. rubidus* and *P. m. sonoriensis*, involving 48 individuals.

(*d*) between *P. e. eremicus* and *P. e. fraterculus*, involving 41 individuals.

Four individuals of each of the two races under comparison were usually used in a single experiment. These eight mice were as nearly comparable as possible, in respect to age, and the two sexes were usually equally represented.

I shall use the term "experiment" in referring to each of these comparative tests referred to in the preceding paragraph. Within an experiment, the individuals belonging to a single race, such as *P. e. eremicus* or *P. m. sonoriensis* will be referred to as a "race group." The term "series" will apply to the combined experiments which comprise one of the four racial comparisons: *sonoriensis-eremicus*, *gambelii-fraterculus*, *rubidus-sonoriensis* and *eremicus-fraterculus*.

As stated above, the daily water-intake of an individual was recorded for the duration of the experiment. At the end of the experimental period, the mean daily water-intake for each individual in the experiment was computed, and this value was divided by the weight of the individual in grams. This gives the mean daily water intake in terms of body weight.[2] The weight of each individual was determined to tenths of a gram at the beginning of each experiment. All the figures for water-intake found in Table I are given in terms of cubic centimeters per day per gram.

In each experiment the mean of these individual values for intake per day per gram was determined for each race group. All comparisons between the two races in a series are based upon the difference between the race-group means within an experiment, these differences being weighted by employing the following formula (Sumner, 1915):

$$M_{dif} = \frac{\sum(m - m')(n\,n')}{\sum(n\,n')},$$

in which m and m' are the two corresponding race-group means within an experiment and n and n' are the numbers of individuals upon which the race-group means are based. The value for M_{dif} is the difference in water intake between the two races under comparison in a series.

The probabilities of the racial differences for a series were computed by the method of McEwen (1929) which is based upon "Student's" Probability Integral. The standard deviations from both race-group means within an experiment are obtained. Then the square root of the sum of the squares of these standard deviations is extracted. The difference between the two race-group means is now divided by this standard deviation. The probability corresponding to this value, on the basis of the number of individuals in each race-group, is then found in McEwen's table. This probability value, P, is subtracted from 1.00, giving the probability that the difference is due entirely to chance. The probability that a racial difference within an entire series is due to chance is obtained by multiplying together the values for 1.00 minus P for each race-group difference. This cumulative value I shall refer to as the "series probability."

[2] The standard of cubic centimeters per day per gram was adopted, in spite of the interesting results reported by Richter and Brailey (1929), who studied the water intake of a group of white rats from the age of 30 days to 160 days. These workers found that increase in water intake with age was closely correlated with increase in body surface, but much less closely with increase in body weight. They state further that daily water intake per unit of surface area was found to be practically a constant value at all ages. An attempt was made, in connection with the present investigation, to calculate water intake in relation to surface, and compare the standard of intake referred to surface with intake referred to weight. The results indicated that any differences depending on race were as clearly demonstrated by the use of the body weight standard as by that of body surface. In no case did the water intake per unit of surface approach a constant value as Richter and Brailey found. In view of these facts it was thought justifiable to neglect the intake-surface relationship and use the standard of cubic centimeters per gram of body weight.

TABLE I

		Sonoriensis				*Eremicus*							
	Exper.	No. Indi-viduals	Mean Daily Water Intake *cc's/gram*	Extremes	σ	No. Indi-viduals	Mean Daily Water Intake *cc's/gram*	Extremes	σ	Difference between Means $M_s - M_e$	$1-P$		
Series *A*	1	4	.268	.183–.325	.055	4	.162	.133–.192	.021	.106	.0263	Weighted mean, *sonoriensis*,	0.174
	2	4	.309	.180–.603	.169	4	.139	.114–.162	.017	.170	.0908	" " *eremicus*,	0.124
	3	8	.128	.100–.176	.035	6	.124	.108–.171	.021	.004	.4072	" difference, $M_s - M_e$,	+0.037
	4	8	.114	.071–.189	.040	8	.108	.092–.155	.018	.006	.3622	Cumulative value, $1-P$,	0.000017
	5	4	.159	.130–.217	.034	5	.107	.087–.165	.029	.052	.0509		
		Gambelii				*Fraterculus*				$M_g - M_f$			
Series *B*	1	4	.116	.061–.171	.047	4	.078	.046–.109	.022	.038	.1404	Weighted mean, *gambelii*,	0.160
	2	4	.131	.101–.195	.037	4	.098	.071–.141	.026	.033	.1482	" " *fraterculus*,	0.117
	3	4	.185	.164–.208	.017	4	.153	.114–.210	.035	.032	.1257	" difference, $M_g - M_f$,	+0.044
	4	4	.145	.126–.160	.013	4	.143	.113–.178	.029	.002	.4622	Cumulative value, $1-P$,	0.0000008
	5	8	.175	.106–.274	.046	8	.118	.082–.197	.033	.057	.0166		
	6	5	.186	.133–.294	.059	4	.109	.101–.127	.010	.077	.0425		
		Rubidus				*Sonoriensis*				$M_r - M_s$			
Series *C*	1	4	.237	.161–.358	.077	4	.372	.155–.534	.182	−.135	.1519	Weighted mean, *rubidus*,	0.200
	2	4	.176	.140–.210	.025	4	.186	.110–.256	.040	−.010	.3703	" " *sonoriensis*,	0.206
	3	4	.190	.157–.222	.023	4	.174	.164–.184	.007	.016	.1683	" difference, $M_r - M_s$,	−0.004
	4	4	.187	.169–.211	.015	4	.161	.146–.181	.013	.026	.0469		
	5	8	.205	.145–.283	.044	8	.172	.112–.290	.069	.033	.1625		
		Fraterculus				*Eremicus*				$M_f - M_e$			
Series *D*	1	4	.067	.041–.089	.017	4	.084	.051–.137	.032	−.017	.2383	Weighted mean, *fraterculus*,	0.117
	2	5	.164	.097–.223	.051	3	.136	.118–.148	.012	.028	.2175	" " *eremicus*,	0.107
	3	8	.118	.082–.197	.033	8	.108	.092–.155	.018	.010	.2504	" difference, $M_f - M_e$,	+0.007
	4	4	.109	.101–.127	.010	5	.107	.087–.165	.029	.002	.4591		

RESULTS

The results (Table I, Figs. 1, 2, 3) show that, in the *sonoriensis-eremicus* series, *sonoriensis* has a higher water intake than *eremicus;* in the *gambelii-fraterculus* series, *gambelii* surpasses *fraterculus.* If the relative magnitude of these differences within the series *A* and *B* are not immediately apparent to the reader, let us note that the weighted difference for the series *A*, 0.037 cc. per day per gram, is 30 per cent of the weighted mean for *eremicus*, and 21 per cent of the weighted mean for *sonoriensis.* Likewise, in series *B*, the weighted difference, 0.044 cc. per day per gram, is 27 per cent of the weighted mean for *gambelii* and 38 per cent of the weighted mean for *fraterculus.* It will be seen from the table that in none of the component experiments in the series *A* and *B*, is the direction of the differences reversed. That is to say, *sonoriensis* is consistently ahead of *eremicus*, and *gambelii* is likewise ahead of *fraterculus* in all cases.

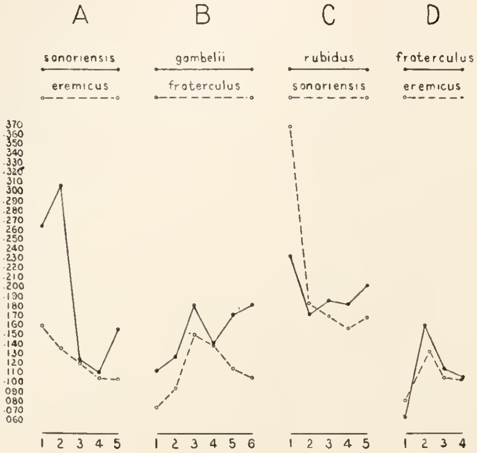
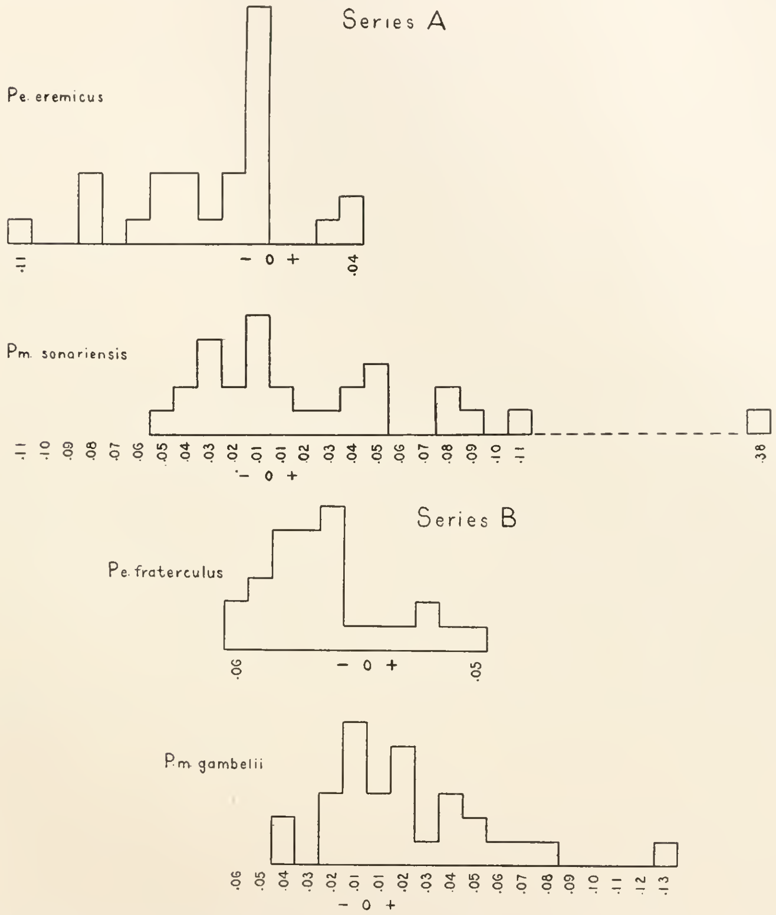


FIG. 1. Graphic representation of the mean value for daily water intake for each experiment in series *A*, *B*, *C* and *D*. Ordinates indicate water consumption in cubic centimeters per gram per day. Abscissae indicate the series and their component experiments.

The series probability for series *A* is 0.000017 and for series *B* is 0.0000008. This means that in the former series there are 1.7 chances in a hundred thousand that the difference is due to chance, and in the latter series, there is less than one chance in a million that the difference is due to chance. We are therefore forced to the conclusion that the differences obtained in these two series are significant ones.

Combining series *A* and *B*, we have an interesting specific comparison, between *Peromyscus maniculatus* (*sonoriensis* and *gambelii*) and *P. eremicus* (*eremicus* and *fraterculus*). The former gives a weighted mean value of 0.167, the latter a weighted mean value of 0.120, the weighted difference being 0.040.



FIGS. 2 and 3. Frequency distributions, based upon the deviation (+ or –) of each individual from the mean value (daily water in cc./grams) of the total population of the experiment to which it belonged. In computing this mean value, the two races in each experiment were thrown together and treated as a single population. Values to the right of the zero line represent positive deviations, while those to the left are negative deviations.

Figure 2 is for series *A* and Fig. 3 is for series *B*.

In series *C* and *D* we find, on the other hand, that the weighted differences between the races concerned are very small. The weighted difference in series *C* shows that *rubidus* is 0.006 cc./gram/day ahead of *sonoriensis*. This difference is but 3 per cent of the weighted means for *rubidus* and for *sonoriensis*. In series *D*, *fraterculus* is 0.007 cc./gram/day ahead of *eremicus*. This weighted difference is about 7 per cent of the weighted mean value for *eremicus* as well as of that for *fraterculus*.

In these two series, likewise, we do not find the same consistency in the direction of the differences as was the case in series *A* and *B*. For instance, in series *C*, in three experiments, *rubidus* is ahead of *sonoriensis*, while in two, *sonoriensis* is ahead of *rubidus*. In series *D*, *fraterculus* is ahead of *eremicus* in three experiments, while in the fourth the direction of the difference is reversed.

The small differences within the two series *C* and *D* and the fact that the component experiments display no constancy in the direction of their differences, lead us to the conclusion that we have demonstrated no significant differences in water intake between *P. m. rubidus* and *P. m. sonoriensis*, nor between *P. e. eremicus* and *P. e. fraterculus*.

The reader will perhaps note that the weighted mean water-intake value for *P. m. rubidus* in series *C* is above that for *P. m. sonoriensis* in series *A*. Also, the *P. m. sonoriensis* value in series *A* is above the *P. m. gambelii* value in series *B*. These differences are not considered valid, inasmuch as they are not based upon comparisons between races which were made at the same time, with animals of comparable age, under the same conditions of temperature and humidity. It is entirely conceivable, had all the possible combinations been tested, that other interesting differences might have been brought out. The particular four comparisons were selected as being of especial interest from the viewpoint of the relation between water intake and the environmental and racial backgrounds of the groups concerned.

In the table will be found under "Extremes," the low and high individual values within each racial group, for each experiment. These low and high values, along with the standard deviations, will give the reader some idea of the high degree of individual variability which is displayed by this material. In this connection see also Figs. 2 and 3. These histograms represent the differences in water-intake in terms of the deviation (+ or −) of each individual from the mean for the experiment to which it belongs. It is obvious that the race which has the lower water intake will display predominately minus deviations and that the race with the higher intake will show predominately plus deviations. This predominance of one sign or the other may be manifested either in the greater frequency or the greater magnitude of the deviations in a given direction, or in both respects.

Discussion

We have seen that in our experiments there are significant differences in water intake within the racial groups which have been the subjects of our experimental comparisons. The differences found are between species, representatives of *P. maniculatus* and *P. eremicus,* and not within a species, either *maniculatus* or *eremicus.*

It is also clear that, since the experimental data are based almost exclusively upon material which was born and reared in captivity, under environmental conditions which were identical for the two species under comparison in any single experiment, these differences in water intake are inherited ones.

A rational explanation of the above facts must involve, not only a consideration of the environments of the parent stocks of the experimental material, but some speculation concerning their phylogenetic backgrounds.

Our experimental data point to the probability that *P. m. gambelii* has a higher water intake than *P. e. fraterculus.* While the greater part of the geographic distribution of *gambelii* embraces a region in which more humid climatic conditions prevail than in the range of *fraterculus,* the range of the former does to a certain degree overlap that of the latter. The two forms occur together in the coastal belt of California, south of Los Angeles, and in the northern coastal region of Lower California. It is from this overlapping region that the parent stocks of experimental material for both races were secured.

It has been indicated in our experiments that *P. m. sonoriensis* has a higher water intake than *P. e. eremicus.* In considering the geographic distribution of these two races, we see that their ranges overlap to a certain degree and that where their ranges do not overlap, similar climatic conditions prevail.

Here we have two cases (*gambelii-fraterculus, eremicus-sonoriensis*), in which two forms belonging to separate species of the genus *Peromyscus* are found in a state of nature living under similar climatic conditions, but as tested in the laboratory, show very definite differences in water intake.

It seems evident then that we must search farther than adaptation to the general climatic environment of a region for an explanation of the differences in water intake which we have found. Perhaps we may find the solution in a consideration of the racial backgrounds of the groups with which we are concerned. From the geographic distribution of the two species of *Peromyscus, maniculatus* and *eremicus,* we may deduce much concerning their probable evolutionary histories. The reader is referred to Osgood's (1909) maps of geographic distribution for *P. maniculatus* and for *P. eremicus*. It will again be noted, as mentioned in the introduction, that the species *maniculatus* has a very widespread distribution. Although it is fairly well represented in the arid and semi-arid southwest, perhaps eight or nine-tenths of the geographic range of this very ubiquitous species is in more humid regions. The species *eremicus*, on the other hand, has a much more limited distribution, being essentially a southwestern form, confined to arid regions of southwestern United States and nearby portions of Mexico.

May we not frame a somewhat speculative explanation of the difference in water intake between *maniculatus* and *eremicus*, on the basis of their probable racial histories deduced from their geographic distributions?

We may suppose that the stem form from which the numerous sub-species of *P. maniculatus* were differentiated had its origin in a relatively humid environment. This assumption is a safe one, since desert habitats for *maniculatus* are exceptional, perhaps ten per cent of its total geographic distribution. After a lapse of time, the group extended its geographic range from its point of origin, some forms migrating to more arid localities. These forms became slowly adapted to the environmental conditions encountered there, but may have retained to some extent the ancestral modes of life.

A similar line of thought may be applicable to the case of *P. eremicus*. We may suppose that the *eremicus* stem-form had its origin in an arid locality and gradually extended its range. Some groups became adapted to a certain extent to less arid climatic conditions but retained many of the characteristics of the ancestral type.

There are observations by various workers concerning the habitat preferences of certain races of *Peromyscus*, which are very interesting in connection with the foregoing discussion of racial history, and also with the experimental results which I have obtained. Dr. F. B. Sumner states it as a matter of practical field experience that when trapping for *Peromyscus* in a given locality, *P. maniculatus* is found more typically in relatively moister habitats, while *P. eremicus* prefers more arid situations. For instance, in certain arid regions, this worker finds that *P. m. sonoriensis* is taken in large numbers along flood plains of rivers and other low-lying ground, where relatively few *P. e. eremicus* are caught. When the trap-line is run up on the arid banks and barren slopes, even a few hundred yards away, *eremicus* may be obtained in large numbers, while *sonoriensis* is scarce. There is a similar relationship between *P. m. gambelii* and *P. e. fraterculus* in the coastal region of Southern California. *Gambelii* is taken in large numbers in grassy valleys and the bottoms of canyons, while *fraterculus* is more often found in abundance on the dry hillsides.

Grinnell (1914) in his "Account of Mammals and Birds of the Lower Colorado Valley" states that *P. m. sonoriensis* is an "abundant inhabitant of bottom lands everywhere" and that "this *Peromyscus* [*P. m. sonoriensis*] has appropriated the river bottom, which, in turn, is tabooed by the two desert species of the region, *eremicus* and [*P. crinitus*] *stephensi*." And of *P. e. eremicus*, the same author says "yet our trapping showed distinct associational preferences. The overflow bottom is evidently rarely invaded, there being but slight overlapping of the habitat of *P. maniculatus sonoriensis*."

It appears then, that *P. e. eremicus*, even though it may occur under the same climatic conditions as *P. m. sonoriensis*, is influenced by its racial background in such a manner as to display a predisposition to a lower water intake than *sonoriensis*, and to seek an immediate habitat which is compatible with this tendency. Likewise, *P. e. fraterculus*, although its geographic range overlaps to a certain extent that of *gambelii*, consumes less water than the latter, because of an inherited predisposition to a more limited water intake. *Fraterculus* and *gambelii* show habitat preferences in accordance with this physiological character.

In view of the fact that no differences in water intake were demonstrated between *P. m. rubidus* and *P. m. sonoriensis*, we may suppose, pending further investigation, that *sonoriensis* has not yet diverged sufficiently from forms such as *rubidus* to display a difference in water intake sufficient to be demonstrated by the somewhat crude methods employed in this investigation. Likewise, since no water-intake difference could be proved between *P. e. eremicus* and *P. e. fraterculus*, it would appear that *fraterculus* is not at present far enough removed from *eremicus* to show a significant difference in water consumption.

Summary

1. Five subspecific groups of *Peromyscus*, representative of the two species, *maniculatus* and *eremicus*, have been tested in the laboratory to determine whether racial differences in water intake exist.

2. Significant differences were found between *P. m. sonoriensis* and *P. e. eremicus*, as well as between *P. m. gambelii* and *P. e. fraterculus*. No significant differences between *P. m. rubidus* and *P. m. sonoriensis*, nor between *P. e. eremicus* and *P. e. fraterculus*, were demonstrated.

3. It is seen that, in these experiments, the only demonstrable differences in water intake are between the two species, *P. maniculatus* and *P. eremicus*, and not within either species. These specific differ-

ences in water intake are interestingly correlated with the geographic and ecological distributions of the two species.

4. Since the experimental data are based almost exclusively upon cage-bred stock, reared in a common environment, these differences appear to be hereditary.

5. Adequate statistical treatment, using McEwen's method, based upon "Student's" Probability Integral, indicates that these differences in water intake are real ones and not due to chance.

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QUANTITATIVE STUDIES IN ION ANTAGONISM

I. Experiments on the Striated Muscle of the Frog

E. GELLHORN

(*From the Department of Animal Biology, University of Oregon, Eugene, Oregon*)

Although the study of ion antagonism goes back to the year 1882 when Ringer published his first paper on ion antagonism in the heart, quantitative study of the subject has just begun. Loeb (1915*b*) suggested the interesting theory that the quantitative relationship in ion antagonism depends upon the function concerned. If ions influence irritability, a linear relation is supposed to exist between the antagonistic ions, while a parabolic relation expresses the antagonism if permeability is concerned. Recently Rubinstein (1928) performed experiments on a polychæte worm (*Fabricia sabella*) and found a parabolic relationship $[K] = a\sqrt{[Na]}$, although there was no proof that this antagonistic action was based upon changes in permeability. It is the writer's opinion that a sharp distinction between the two groups as suggested by Loeb is not possible at the present time. Changes in the behavior of the surface of cells may often initiate changes in permeability (Gellhorn, 1930). In spite of this, the ingenious idea of Loeb, that the quantitative study of ion antagonism may reveal different kinds of antagonism and may finally lead to a better understanding of that problem, merits further investigation. In contrast to the work of Loeb (1915*a*) on *Balanus* larvæ and that of Rubinstein (1926) on *Fabricia*, in which several tissues were involved, the writer has considered it preferable to examine ion antagonism as it affects one single structure. This is possible in muscle, for Kato (1929) has shown in histological studies that the antagonistic ions act upon the same anatomical structure.

It is apparent from the investigations of Overton (1904) that the irritability of striated muscle depends chiefly upon NaCl, KCl, and $CaCl_2$. Therefore a quantitative study of ion antagonism was carried out between these ions. The effect of the trace of $NaHCO_3$ added to the Ringer's solution to preserve neutrality was not taken into consideration.

METHOD

The experiments were chiefly performed on the sartorius muscle of *Rana esculenta* between January and April, 1930. Frogs of medium size, which did not vary more than 10 per cent in weight, were chosen. The sartorii were prepared carefully in the usual manner and suspended between platinum electrodes. The muscles were kept in the salt solution, which was aerated with purified oxygen. At intervals of about thirty minutes the threshold of the muscle was determined after having removed the muscle from the liquid. The interrupted current of an induction coil, the primary circuit of which was supplied with one dry cell (1.5 volts), was used. In a series of preliminary experiments it was found that if at constant NaCl and $CaCl_2$ content the influence of increasing KCl concentration was investigated during the observation time of three hours, a sharp distinction between two groups of KCl effects could be made. In the first group, containing

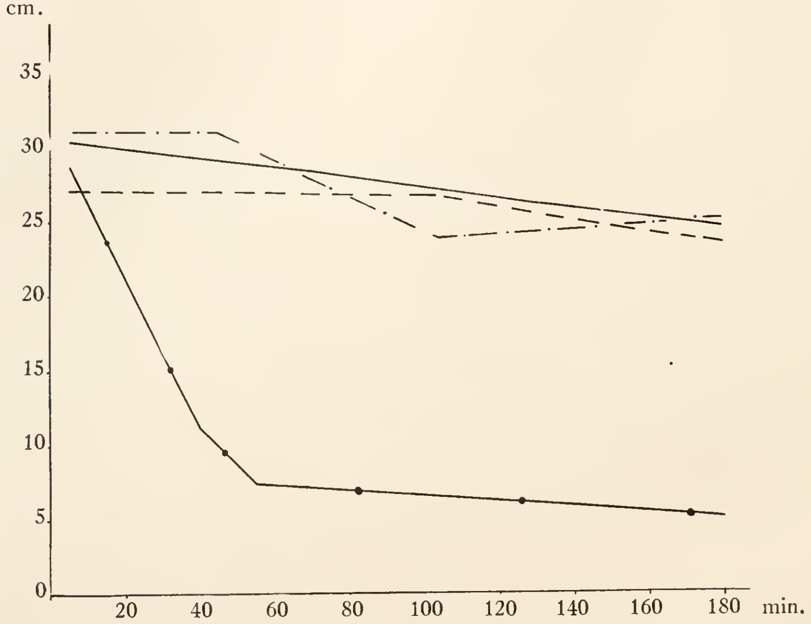

FIG. 1. The influence of the variation of KCl—NaCl and $CaCl_2$ being constant —upon the irritability of the sartorius.

Ordinate: threshold in cm. distance between primary and secondary coil.

Abscissa: time in minutes.

0.042 M NaCl + 8.1 M $\times 10^{-4}$ $CaCl_2$ + 11.3 M $\times 10^{-4}$ KCl ————
" " + 17 M $\times 10^{-4}$ KCl — — — — —
" " + 20 M $\times 10^{-4}$ KCl —·—·—·—·
" " + 23 M $\times 10^{-4}$ KCl —●—●—●—

the lower KCl concentration, the threshold increased very gradually within three hours and the final value was indicated by a distance of at least 20 cm. between primary and secondary coils. In the greater concentrations, however, the irritability decreased very rapidly and fell far below 20 cm. The initial value, which varied between 25 and 35 cm. distance, had no influence on this typical behavior. Thus it was possible to determine exactly the threshold of the KCl effect which just leads to a loss of irritability below 20 cm. distance. In about 200 experiments, there were very few cases where the classification was doubtful. In such cases several repetitions of the experiments gave decisive results. A typical example illustrating the accuracy of the method is reproduced in Fig. 1. Further on it is shown in Fig. 2 that the antagonistic action of $CaCl_2$ can be determined exactly in the same manner.

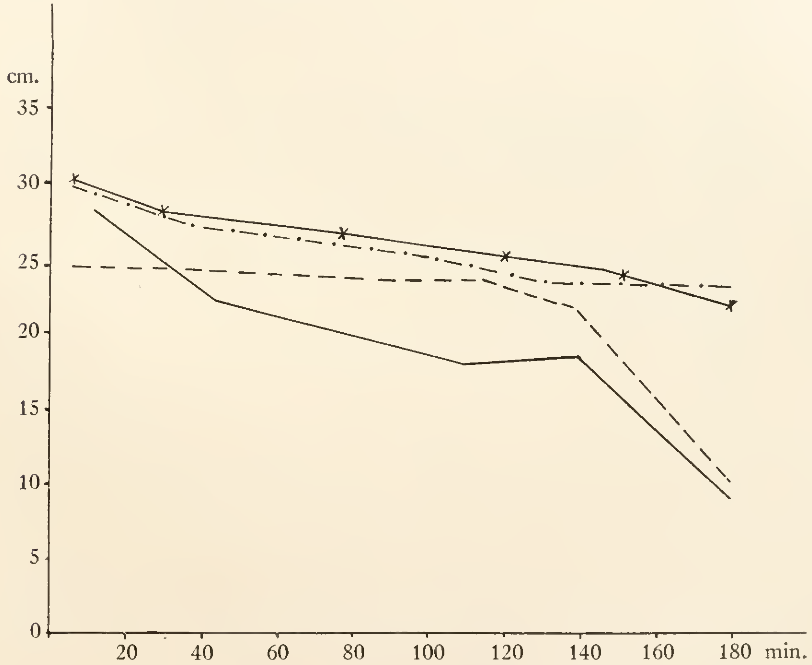

FIG. 2. The influence of variation of $CaCl_2$—NaCl and KCl being constant—upon the irritability of the sartorius.

Ordinate and abscissa as in Fig. 1.

0.084 M NaCl + 84 M × 10^{-4} KCl + 46.57 M × 10^{-4} $CaCl_2$ —————
" " 48.6 " — — — —
" " 50.62 " —·—·—·—
" " 52.65 " —x—x—x—

The solutions used had a freezing point of Δ = − 0.39. They were 0.113 M NaCl, 0.113 M KCl and 0.081 M $CaCl_2$. Greater concentrations of NaCl were obtained with different amounts of 1 per cent NaCl. The amount of liquid used was always 20 cc.

I. *The Antagonism between Na and K*

In a series of experiments the dependence of the KCl effect upon the NaCl concentration was studied. The $CaCl_2$ concentration, $8.1 \text{ M} \times 10^{-4}$, was constant in all experiments. There were two ways of varying the NaCl concentration: first, by replacing a part of the NaCl solution by isotonic glucose solution, thus avoiding changes in the osmotic pressure for all solutions containing less NaCl than corresponds to the isotonic concentration; second, without the

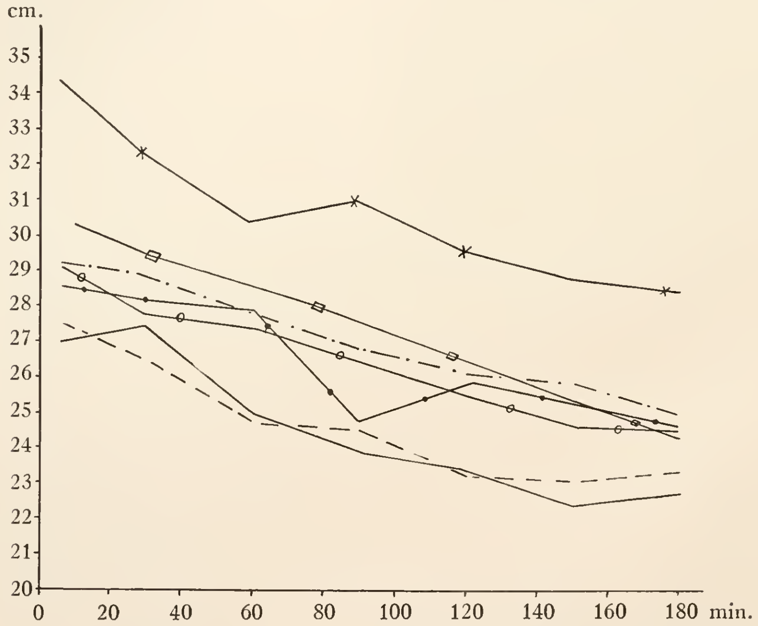

FIG. 3. The influence of variation of NaCl—KCl and $CaCl_2$ being constant—upon the irritability of the muscle. Each solution contains $5.65 \text{ M} \times 10^{-4}$ KCl + $8.1 \text{ M} \times 10^{-4}$ $CaCl_2$.

Ordinate and abscissa as in Fig. 1.

0.042 M	NaCl	—□—□—
0.056 M	"	— — — —
0.071 M	"	————
0.08 M	"	—x——x—
0.09 M	"	—o——o—
0.113 M	"	—·—·—·—
0.127 M	"	—●——●—

addition of any non-electrolytes. The experiments were performed in both ways and it was found that glucose even in small amounts in the presence of NaCl is not indifferent to the muscle. The effective minimal concentration of KCl was greater with the addition of glucose than without it. This shows that glucose protects the muscle to a certain extent from the harmful effect of KCl. Similar observations were made in a study of the KCl contracture in muscle (Gellhorn, 1926). Because the osmotic pressure can be maintained constant for various concentrations of NaCl only by the addition of varying amounts of non-electrolytes, the effective minimal concentration of KCl would also be dependent upon the sugar concentration. In order to avoid these complications, salt solutions without non-electrolytes were used in spite of differences in the osmotic pressure. It is apparent from Fig. 3 that it was possible to study the effect of NaCl concentration between the values 0.042 M and 0.127 M because the threshold of the muscle remained above 20 cm. for three hours in spite of the osmotic differences.

Table I contains data from a series of experiments in which the dependence of the minimal effective KCl concentration upon the NaCl concentration was studied. The experiments show the great accuracy with which this value can be determined. It is:

Concentration KCl ($M \times 10^{-4}$)	for M NaCl
23	0.042
43	0.056
51	0.071
68	0.092
85	0.113

One recognizes that with increasing NaCl concentration the KCl concentration must also increase in order to become effective. There is therefore an antagonism between NaCl and KCl. The quantitative relationship is to be seen in Fig. 4. It shows that there is a linear dependence of [K^+] upon [Na^+] within the limits of 0.049 M NaCl and 0.113 M NaCl. Only in the extreme NaCl concentrations are small deviations from the straight line observed. They are probably due to a greater sensitivity of the muscle to KCl because of the extremely abnormal osmotic pressure. Therefore, further experiments were restricted to the range in which the linear dependence is exactly correct. According to these experiments the ratio of Na to K is constant as may be seen from Table II.

TABLE I

The Dependence of the KCl Effect upon the NaCl Concentration

Group I. 0.042 M NaCl + 0.00081 M $CaCl_2$

$M \times 10^{-4}$ KCl concentration	5.7	11.3	17	20	23	26
Threshold in cm. after 5′	28.0*	30.4	27.1	31.1	29.0	26.4
" " " " 180′	21.8	24.6	23.5	24.7	7.3†	12.0

Group II. 0.056 M NaCl + 0.00081 M $CaCl_2$

$M \times 10^{-4}$ KCl concentration	34.0	40	43	45
Threshold in cm. after 5′	29.4	31.3	31.0	31.4
" " " " 180′	26.9	23.0	6.3	7.9

Group III. 0.071 M NaCl + 0.00081 M $CaCl_2$

$M \times 10^{-4}$ KCl concentration	48	50	51	54	57	68
Threshold in cm. after 5′	31.5	35.5	34.5	32.0	32.1	36.5
" " " " 180′	22.2	21.8	14.5	4.5	7.5	5.7

Group IV. 0.092 M NaCl + 0.00081 M $CaCl_2$

$M \times 10^{-4}$ KCl concentration	62	65	68	71
Threshold in cm. after 5′	27.4	27.3	27.0	26.8
" " " " 180′	26.0	20.5	9.0	7.5

Group V. 0.113 M NaCl + 0.00081 M $CaCl_2$

$M \times 10^{-4}$ KCl concentration	79	82	85
Threshold in cm. after 5′	36.5	27.2	27.5
" " " " 180′	23.2	20.5	9.0

* The numbers represent the threshold of the muscle measured by the distance between primary and secondary coil.

† The underlining indicates that the threshold has fallen below 20 cm. distance.

TABLE II

Ratio of Na to K for Different Na Concentrations

M NaCl	0.042	0.049	0.056	0.071	0.085	0.092	0.099	0.113	0.127
$\frac{M\ NaCl}{M\ KCl}$	1.87	1.33	1.39	1.36	1.36	1.35	1.34	1.34	1.41

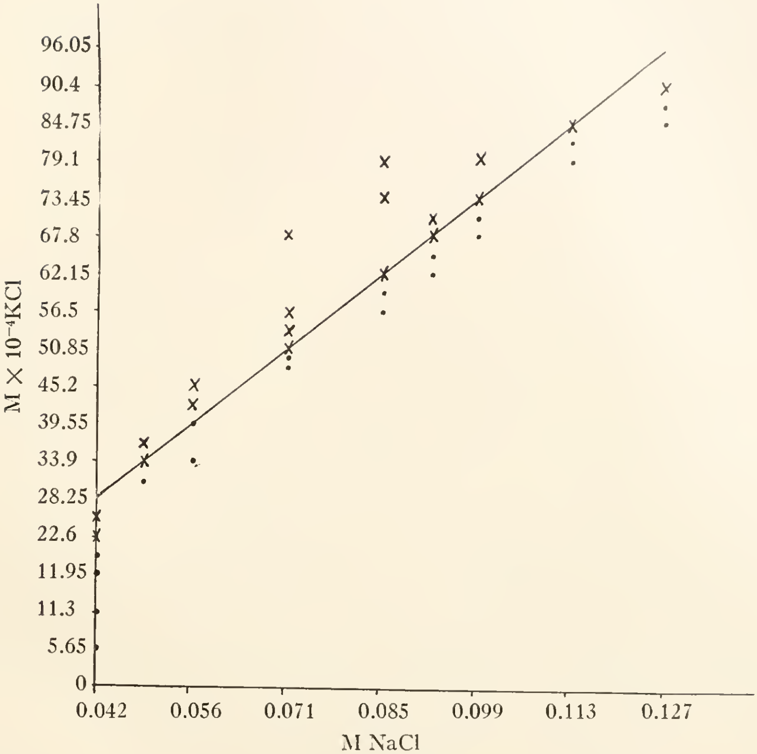

FIG. 4. The influence of the Na concentration upon the K effect.
Abscissa: NaCl concentration.
Ordinate: KCl concentration.

x = Rapid loss of irritability.
· = Preservation of irritability.

II. *The Antagonism between K and Ca*

The solutions, the effects of which on irritability of the sartorius muscle were detailed in Part I, contained 8.1 M $\times 10^{-4}$ $CaCl_2$. Because it is known that Ca is the antagonist of K in muscle, the K effect in our experiments was balanced by NaCl + $CaCl_2$. In order to learn the quantitative relationship between K and Ca, the KCl concentration was varied and that $CaCl_2$ concentration which just sufficed to maintain the irritability above the 20 cm. threshold was determined. In these experiments the NaCl concentration was constant (0.071 mol.). As shown in Fig. 4, 8.1 M $\times 10^{-4}$ $CaCl_2$ was able to equilibrate the effect of 50 M $\times 10^{-4}$ KCl, but at the same $CaCl_2$ concentration 51 M $\times 10^{-4}$ KCl led to a rapid decrease in irritability. In a series of experiments the corresponding $CaCl_2$

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concentrations were determined when the solution contained 68 M × 10^{-4} KCl and 85 M × 10^{-4} KCl. The results were as follows:

TABLE III

The Ca → K Antagonisms in 0.071 *mol. NaCl Solution*

M × 10^{-4} KCl..........	50	68	85
M × 10^{-4} $CaCl_2$.........	8.1	40.5	72.9

As Table III shows, the increasing concentration of KCl requires an increasing concentration of $CaCl_2$. Hence a linear relationship seems to exist between the two salts, since equal amounts of $CaCl_2$ above the basic value of 8.1 M × 10^{-4} antagonize approximately equal increases of KCl. But there is a very characteristic limit for this ratio. A KCl concentration which is higher than 91 M × 10^{-4} cannot be balanced even with the greatest concentration of $CaCl_2$.

On the other hand, further investigations showed that the antagonistic action of $CaCl_2$ against KCl is relatively greater below 8.1 M × 10^{-4} $CaCl_2$ than it is above it. Between 50 and 80 M × 10^{-4} KCl about 32.4 M × 10^{-4} $CaCl_2$ is able to offset the effect of 17 M × 10^{-4} KCl. That corresponds to the relationship, 0.001 M $CaCl_2$ to 5.25 M × 10^{-4} KCl. But as Table IV shows, below 8.1 M × 10^{-4} $CaCl_2$ the relationship is 0.001 M $CaCl_2$ to 27.7 M × 10^{-4} KCl.

TABLE IV

The Ca → K Antagonism in 0.07 *mol. NaCl Solution*

M × 10^{-4} KCl conc.........	34	39	44	50
M × 10^{-5} $CaCl_2$ conc.......	20.25	40.5	60.75	81

These data lead to the conclusion that the Ca → K antagonism is represented by two straight lines forming an angle with each other. However, as Tables III and IV indicate, there is no constancy of the ratio of K to Ca when the $CaCl_2$ concentration is either below or above 8.1 M × 10^{-4}. The difference in the quantitative behavior of the Na → K antagonism and that between Ca → K can be expressed mathematically. The equation of the straight line $y = ax + b$ holds for both antagonisms, but since in the former case b equals 0, $a = y/x =$ const., while in the Ca → K relationship b has a negative value. The constants of the experiments of Table III are $a = 1.9$, $b = -87.9$; those of Table IV, $a = 0.36$, $b = -9.7$.

Summing up these results and those of Part I, it may be said that Na and Ca ions are antagonistic to K. Leaving out of consideration the case in which the $CaCl_2$ concentration is below 8.1 M × 10^{-4}, the data show that 11.3 M × 10^{-4} KCl can be equilibrated by 20.25 M × 10^{-4} $CaCl_2$. Furthermore, it is apparent from Fig. 4 that 0.014 M NaCl balances 11.3 M × 10^{-4} KCl.

III. *The Theoretical Calculation of the Sodium, Potassium, and Calcium Antagonism and its Experimental Proof*

It was pointed out that the results showed the relationship 0.014 M NaCl → 11.3 M × 10^{-4} KCl and 20.25 M × 10^{-4} $CaCl_2$ → 11.3 M × 10^{-4} KCl in which the arrow indicates that the first salt offsets the effect of the second one. On the other hand, the experiments reproduced in Fig. 4 show the minimal effective KCl concentration for different concentrations of NaCl in the presence of 8.1 M × 10^{-4} $CaCl_2$. Therefore, if the suppositions are correct, it should be possible to calculate the minimal effective KCl concentration if either the NaCl or the $CaCl_2$ concentration or both vary.

A series of experiments was performed in order to check the theoretical calculations and experimental facts. The experimental results are reproduced in Table V. The composition of the solutions is calculated according to the data described above. The $CaCl_2$ concentration of these solutions is supposed to be just insufficient to offset the KCl effects. Therefore it was expected that the addition of a small amount of $CaCl_2$ would warrant the maintenance of irritability above the 20 cm. threshold. Solutions which had this effect are marked with +, solutions causing rapid loss of irritability below 20 cm. threshold are marked with −. The calculated and the experimentally determined values check very well and show again that a linear dependence of KCl upon NaCl and $CaCl_2$ is characteristic of the ion antagonism in striped muscle.

In order to check these experiments still another series of experiments was performed on the gastrocnemius muscle of *Rana esculenta.* The muscle weight varied between .45 gram and .50 gram. Because these muscles are larger and the specific surface smaller, it was to be expected that they would be less sensitive to KCl than the sartorius muscles with their large specific surface. Correspondingly, it was found necessary to extend the time of observation to four hours in order to find differences in the irritability. Under these conditions the concentration of KCl was determined which just led to a marked decrease in irritability if NaCl and $CaCl_2$ were constant. This is a typical example:

M NaCl	0.084	0.084	0.084	0.084
M × 10^{-4} KCl	68	71	74	80
M × 10^{-4} $CaCl_2$	18.23	18.23	18.23	18.23
Threshold after 4 hours, in cm.	31.6	32.6	23.8	11.0

TABLE V

The Antagonism between Na, K and Ca

+ = maintenance of irritability above the 20 cm. threshold.
− = rapid decrease of irritability; threshold below 20 cm.

					$M \times 10^{-4}$ $CaCl_2$	
					Observed	Calculated
Group I. 0.056 M NaCl + 0.0051 M KCl						
$M \times 10^{-4}$ $CaCl_2$	28.35	30.38	32.4		28.35	28.35
Irritability	−	+	+			
Group II. 0.056 M NaCl + 0.0068 M KCl						
$M \times 10^{-4}$ $CaCl_2$	58.73	60.75	62.78		60.75	58.73
Irritability	−	−	+			
Group III. 0.084 M NaCl + 0.0068 M KCl						
$M \times 10^{-4}$ $CaCl_2$	16.2	18.23	20.25		16.2	18.23
Irritability	−	+	+			
Group IV. 0.084 M NaCl + 0.0085 M KCl						
$M \times 10^{-4}$ $CaCl_2$	46.57	48.6	50.62	52.65	48.6	48.6
Irritability	−	−	+	+		
Group V. 0.092 M NaCl + 0.0085 M KCl						
$M \times 10^{-4}$ $CaCl_2$	36.45	38.48	40.5		36.45	38.48
Irritability	−	+	+			
Group VI. 0.098 M NaCl + 0.0085 M KCl						
$M \times 10^{-4}$ $CaCl_2$	28.35	30.38	32.4		28.35	28.35
Irritability	−	+	+			
Group VII. 0.098 M NaCl + 0.0102 M KCl						
$M \times 10^{-4}$ $CaCl_2$	52.65	56.70	60.75	64.80	52.65	58.70
Irritability	−	+	+	+		
Group VIII. 0.098 M NaCl + 0.0097 M KCl						
$M \times 10^{-4}$ $CaCl_2$	40.5	44.55	48.6	52.65	40.5	48.6
Irritability	−	+	+	+		
Group IX. 0.113 M NaCl + 0.0097 M KCl						
$M \times 10^{-4}$ $CaCl_2$	24.3	28.35	32.4		24.3	28.35
Irritability	−	+	+			

In this series 74 $M \times 10^{-4}$ KCl was the smallest concentration which was effective. In the same way, the KCl concentrations in the presence of different amounts of NaCl and $CaCl_2$ which led to the threshold below 25 cm. distance between primary and secondary coils were determined. The results have been reproduced in Table VI.

They indicate that the calculated values are generally a little smaller than the observed ones, but that this is almost a constant error, showing that the same relations hold as well for the gastrocnemius as for the sartorius.

TABLE VI

The Antagonism between Na, K, and Ca (Experiments on Gastrocnemius)

No.	M NaCl	M × 10^{-4} $CaCl_2$	M × 10^{-4} KCl	
			Observed	Calculated
1	0.056	28	54	51
2	0.056	59	71	68
3	0.071	18	57	57
4	0.071	49	79	76
5	0.084	18	74	68
6	0.084	49	91	85
7	0.092	38	91	85
8	0.098	28	88	85
9	0.098	49	99	97
10	0.113	28	102	97

IV. *The Limits of the Antagonistic Efficiency of $CaCl_2$*

In the last paragraph the possibility of calculating the minimal effective KCl concentration for different amounts of NaCl and $CaCl_2$ was indicated and a fairly good agreement was shown between experimental and calculated values. In these experiments the difference between the effect of NaCl and $CaCl_2$ in antagonizing KCl was of quantitative nature. But further experiments showed that this holds only within certain limits because the maximal concentration of KCl which can be balanced by $CaCl_2$ is determined by the concentration of NaCl. In Table VII it is shown that the maximal concentration of KCl which could be balanced by $CaCl_2$ varied from 71 to 108 M × 10^{-4} if the NaCl concentration varied.

TABLE VII

Limits of the Antagonistic Action of Ca toward K in Dependence upon the NaCl Concentration

M NaCl concentration	Maximal tolerated K concentration (M × 10^{-4})
0.056	71
0.071	91
0.084	102
0.098	108

The experiments make it probable that NaCl, although comparatively weaker than $CaCl_2$ in antagonizing KCl, exerts in part a specific influence. In this respect it cannot be replaced by $CaCl_2$. It might be thought that variation in the osmotic pressure might influence these results. But as shown in Fig. 4, the linear relationship between Na and K, in spite of a great variation of osmotic pressure, makes this view rather improbable. The direct proof is hardly possible because, as already shown, non-electrolytes such as dextrose are not indifferent to the muscle but reduce its susceptibility to KCl.

Towards the upper limit determined by the NaCl concentration the antagonisms followed the quantitative rules described above. Much greater concentration of $CaCl_2$ than that required by our equations had almost no influence. From Table VIII it is apparent that in 0.056 M NaCl the maximal tolerated concentration of KCl increased from $71\ M \times 10^{-4}$ to $74\ M \times 10^{-4}$ if greater amounts of $CaCl_2$ than calculated were used. For the other concentration of NaCl the limiting concentration of KCl was identical no matter whether $CaCl_2$ was added in calculated amounts or in larger ones. Only in 0.098 M NaCl the limiting KCl concentration was sometimes increased from $108\ M \times 10^{-4}$ to $113\ M \times 10^{-4}$ if greater amounts of $CaCl_2$ were added.

TABLE VIII

Determination of the Maximal Concentration of KCl which can be Balanced by $CaCl_2$

		If added in calculated amounts			If added in far greater amounts		
M NaCl 0.056	$M \times 10^{-4}$ KCl	71	74	74	74	80	
	$M \times 10^{-4}$ $CaCl_2$	69	75	79	162	180	
	Irritability	+*	−	−	+	−	
M NaCl 0.071	$M \times 10^{-4}$ KCl	91	97		97	102	113
	$M \times 10^{-4}$ $CaCl_2$	83	93		162	162	202
	Irritability	+	−		−	−	−
M NaCl 0.084	$M \times 10^{-4}$ KCl	102	108		108		
	$M \times 10^{-4}$ $CaCl_2$	83	95		126		
	Irritability	+	−		−		
M NaCl 0.098	$M \times 10^{-4}$ KCl	108	113		113		
	$M \times 10^{-4}$ $CaCl_2$	73	83		122		
	Irritability	+	−		+ or −		

* + = preservation of irritability above the 20 cm. threshold.
− = loss of irritability below the 20 cm. threshold.

V. *The Antagonism Na → Ca*

In order to analyse completely the relationship between Na, K, and Ca, experiments were carried out to decide whether still other antagonisms exist than those described above. It was also found that $CaCl_2$ can reduce the irritability of the sartorius muscle if added in a sufficient amount. But, of course, the quantitative difference between the K and the Ca effect is very marked because one needs far larger amounts of $CaCl_2$ than of KCl in order to reduce the irritability of the muscle. On the other hand, the effective concentration of $CaCl_2$ depends upon the NaCl concentration in both cases and increases with increasing NaCl concentration (Table IX). While in a 0.042 M NaCl solution only 81 M $\times 10^{-4}$ $CaCl_2$ is required in order to reduce the irritability of the muscle below the 20 cm. threshold, three times as much is necessary in order to exert the same effect in a 0.056 M NaCl solution. The corresponding concentration in a 0.07 M NaCl is far greater, but the latter was not determined because of the interference with an increase of the osmotic pressure.

TABLE IX

The Na → Ca Antagonism. The effect of different $CaCl_2$ concentrations on irritability.

	M $\times 10^{-4}$ $CaCl_2$				
0.042 M NaCl					
5.7 M $\times 10^{-4}$ KCl	8.1	40.5	60.75	81	101.25
Irritability	+	+	+	−	−
0.056 M NaCl					
5.7 M $\times 10^{-4}$ KCl	162.0	182.25	202.5	222.75	243.0
Irritability	+	+	+	+	−
0.071 M NaCl					
5.7 M $\times 10^{-4}$ KCl	299.7				
Irritability	+				

The experiments prove that Na is an antagonist of K and Ca because the effective concentrations of K and Ca increase with increasing concentrations of Na. Experiments of Part II showed that Ca is the antagonist of K, since the effective concentration of K varied in the same direction with varying Ca concentration. It still remains a question whether K also is the antagonist of Ca. If this be the case, it was to be expected that the addition of K could reduce the harmful effect of $CaCl_2$ or that it would increase the effective concentration of the latter. Neither was the case.

SUMMARY

The irritability of the sartorius muscle of *Rana esculenta* has been studied in different mixtures of NaCl, KCl, and $CaCl_2$. The method

permits the detection of very small differences in the KCl effect. Usually the minimal effective KCl and $CaCl_2$ concentration is determined which leads to a rapid loss in irritability. The dependence of the KCl effect upon the NaCl and the $CaCl_2$ concentration is represented by a straight line. Concerning the antagonism between Na and K the result is that the ratio Na : K is constant. That does not hold for the antagonism between K and Ca although this also corresponds to a straight line. But in the latter case the relation is represented by the equation $y = ax + b$ and b has a definite value, while in the first case $b = 0$ and therefore $a = y/x$. The quantitative results show that within certain limits 0.014 M NaCl has the same antagonistic value against KCl as 0.00081 M $CaCl_2$. Therefore, it is possible to predict the behavior of the muscle in any other solution if the antagonism has been investigated for one single mixture of these three salts. The results agree rather exactly with this assumption. There is, however, a very characteristic limit of the validity of these equations. It is found that the NaCl concentration determines the highest KCl concentration, the effect of which can still be offset by the addition of $CaCl_2$. It is higher the greater the NaCl concentration. Therefore the antagonistic action of Na against K is not only quantitatively but also qualitatively different from the antagonistic effect of Ca against K.

An antagonism between Na and Ca was also found. The complete analysis leads to the qualitative formula: $\begin{array}{ccc} \mathrm{Na} & \rightarrow & \mathrm{K} \\ \searrow & \mathrm{Ca} & \nearrow \end{array}$ in which the arrow indicates the cation which is able to balance the toxic effect of another cation.

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FURTHER STUDIES UPON THE NERVE SUPPLY AND FUNCTION OF SUPERNUMERARY GRAFTED LIMBS

S. R. DETWILER AND G. E. McKENNON

Department of Anatomy, College of Physicians and Surgeons
Columbia University

Introduction

Evidence has accumulated from former experiments to show that the anterior limb rudiment of *Amblystoma,* when grafted four segments caudal to the normal position (autoplastic grafts), may receive one or more of the normal brachial (third, fourth and fifth) nerves. Typically, the grafted limbs are supplied by the fifth (brachial), sixth and seventh nerves. The coördinated activities which such limbs display have been shown to be due to their connection with the normal brachial reflex mechanism through the fifth nerve, for when this nerve is sectioned, leaving the sixth and seventh intact, coördinated activities cease (Detwiler and Carpenter, 1929).

It has been reported also that, when the anterior limb is left intact and an additional forelimb is grafted just caudal to the intact normal (homoplastic graft), the graft never receives any of the normal brachial nerves (third, fourth and fifth), and its function is defective and uncoördinated (Detwiler, 1920, 1925). The supernumerary limb under such conditions was found to be innervated most frequently by the sixth, seventh and eighth spinal nerves (Fig. 1), a fact which explains the non-coördinated motility, since it has been shown that some connection with the brachial region of the cord is essential for coördinated movements.

When the anterior limb rudiment is grafted the distance of four segments caudal to the normal site (autoplastic graft) but under conditions allowing for regeneration of a limb in the orthotopic position; the brachial nerves may become redistributed to both limbs. Typically the fifth grows caudally to the graft, whereas the third and fourth supply the orthotopic regenerant. The characteristic caudal growth of the fifth nerve to the graft has been interpreted as a growth response to an attractive influence which is apparently stronger in the rapidly growing grafted appendage than in the regenerating one (Detwiler, 1925, p. 486).

In the earlier experiments, as reported above, it was found that supernumerary limbs, when grafted just caudal to the normal intact limb, did not receive any nerves from the normal brachial region of the cord. It was thus concluded that the presence of the normal limb, by reason of its proximity to the growing brachial nerves, and the attractive influence which it apparently exerts, is sufficient to prevent these nerves from supplying any additional rudiment implanted in the adjacent field. These conclusions have become subject to modification, however, as a result of recent experiments of the same type, since we have obtained a number of cases with coördinated activities in super-

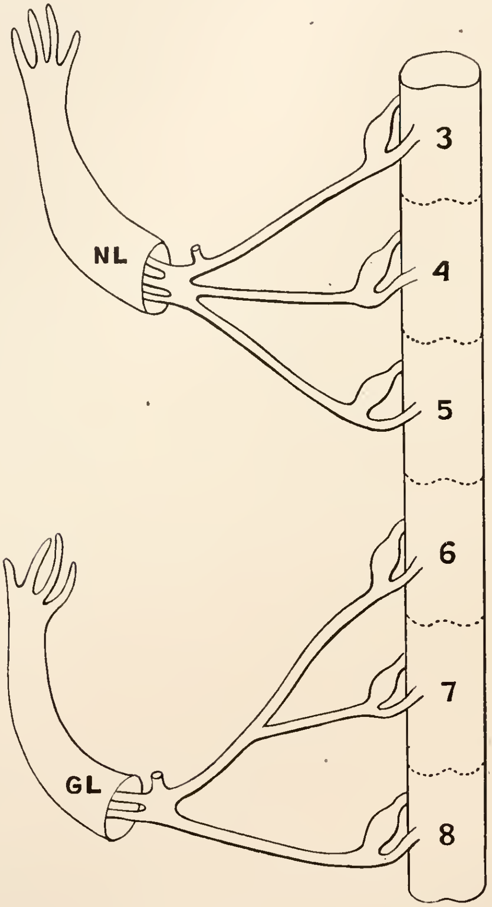


Fig. 1. Diagrammatic plan of segmental nerve contribution (third, fourth and fifth spinal nerves) to the normal anterior limb (*NL*), and to a supernumerary limb (*GL*) (sixth, seventh and eighth spinal nerves), when grafted four segments caudal to the normal limb position.

numerary limbs grafted close to the normal, and the coördinated function has been shown again to be due to the connection of the graft with the brachial correlation mechanism through the fifth (brachial) nerve.

EXPERIMENTAL

During the operating season in 1928 we performed fifty experiments in which a supernumerary limb rudiment was grafted, with normal (dorso-dorsal) orientation, caudal to the intact rudiment so that the graft centered ventral to the eighth somite. This brings the center of the graft just four segments caudal to the center of the normal limb disc, and a space approximately the width of one somite separates the two rudiments (Fig. 3). The operations were performed upon embryos in the tail-bud stage (Harrison's stages 28, 29).

The results of the experiments which are given in Table 1, *A*, show a higher percentage of single limbs than is usually obtained in heterotopic dorso-dorsal grafts. Whereas most of the grafted limbs, whether single or reduplicated, gave evidence of function upon tactile stimulation, there were seven cases in which the grafted appendage functioned in coördination with the normal limbs. In such cases the movements in the graft and in the intact limb of the same side were homologous and synchronous. This phenomenon has been observed and discussed previously (Detwiler, 1925, Weiss, 1924, 1928).

TABLE I

Showing Results of Grafting a Supernumerary Right Anterior Limb Rudiment Four Segments Caudal to the Normal Position (Figs. 3 and 5)

	Orientation	
	A	B
	Normal (Dorso-dorsal)	Inverted (Dorso-ventral)
No. of Operations	50	60
Positive Cases	48	58
Single Limbs	38	42
Reduplications	10	11
Abortive Limbs	2	16
Limbs with Coördinated Function	7	1

Six of the seven cases exhibiting coördinated activities of the grafted limb were sectioned serially for a study of the nerve supply. The results of this study are given in Table II, *A*, where it can be seen that

TABLE II *

Showing Segmental Nerve Supply to Supernumerary Limbs Grafted Caudal to the Right Intact Anterior Limb (Figs. 2, 3, 4, 5)

Group	Case	Orientation of Graft	Distance Caudal to Normal Limb Site	Segmental Nerve Supply to Normal Limb	Segmental Nerve Supply to Supernumerary Grafted Limb
A	4	Dorso-dorsal	4 Segments	3, 4,	5, 6
	5	" "	4 "	3, 4	5, 6, 7
	7	" "	4 "	3, 4, 5	5, 6, 7, 8
	43	" "	4 "	3, 4	5, 6
	49	" "	3 "	3, 4	5, 6
	50	" "	4 "	3, 4, 5	5, 6, 7
B	12	Dorso-ventral	3½ Segments	3, 4, 5	6
	24	" "	2 "	3, 4, 5	6
	25	" "	4+ "	3, 4, 5	7, 8
	28	" "	3 "	3, 4, 5	5, 6
	39	" "	4 "	3, 4, 5	6, 7, 8, 9
	42	" "	3+ "	3, 4, 5	6

* The normal brachial plexus is made up of the third, fourth and fifth spinal nerves.

in every case the supernumerary grafted limb received contribution from the fifth (brachial) nerve. In four of the six cases the entire fifth nerve grew caudally to the graft, whereas in the other two cases the fifth nerve bifurcated so that both normal and grafted limbs were supplied by this nerve. A graphic reconstruction of the segmental nerve contribution to the normal limb and the grafted limb in cases 7, 49 and 50 are shown in Figs. 6, 7 and 8 respectively.

It is to be noted also from the table that in case 49, the final position of the grafted appendage was only three segments caudal to the normal intact limb.

With these unlooked-for results, we carried out during the operating season of 1929, sixty experiments in which the limb was grafted to the same position, but with inverted (dorso-ventral) orientation (Fig. 5 and Table I, *B*). Since the bulk of the limb tissue is concentrated in the antero-dorsal quadrant of the limb disc (Fig. 3), it becomes apparent that when the graft is inverted, the distance between the outgrowing fifth nerve and the concentrated mass of limb-forming cells is considerably increased as compared with the conditions in dorso-dorsal orientations (*cf.* Figs. 3 and 5).

The question arose as to whether under these circumstances (with inverted discs) the fifth nerve would grow caudally to the graft as frequently as in the experiments with dorso-dorsal orientation.

The results of the experiments are given in Table I, *B*. In 58 positive experiments there were six cases in which the graft was capable of considerable function, but only one case with definite coördinated movements.

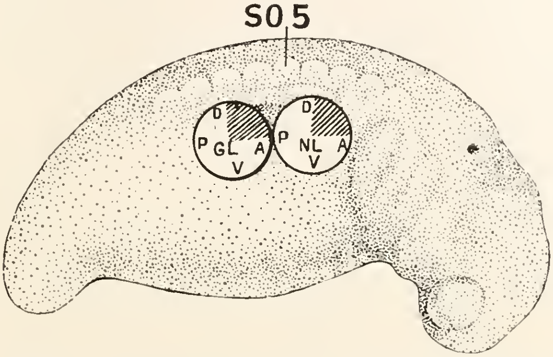


FIG. 2. Drawing of *Amblystoma* embryo, showing supernumerary anterior limb rudiment (*GL*) grafted (with normal orientation) so as to center ventral to the seventh somite. *NL*, normal anterior limb rudiment; *D*, dorsal; *V*, ventral; *P*, posterior; *A*, anterior; *SO*5, fifth somite. The shaded regions indicate the quadrants of the rudiments in which the main mass of the limb-forming cells is concentrated. × 12.

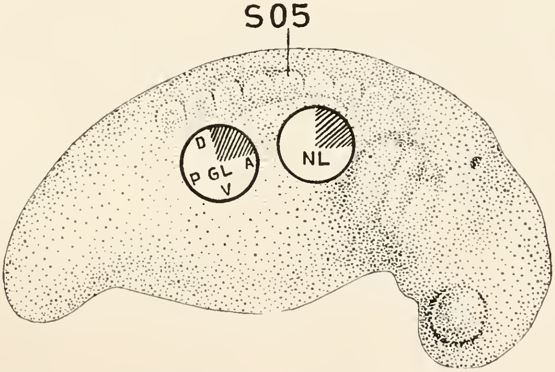


FIG. 3. Drawing of *Amblystoma* embryo showing supernumerary limb (*GL*) grafted (with normal orientation) so as to center ventral to the eighth somite. *NL*, normal anterior limb rudiment; *SO*5, fifth somite. × 12.

The results of a study of the nerve contribution to the six cases selected are given in Table II, *B*. Here it is seen that the one case (No. 28) which definitely displayed coördinated movements, received a branch of the fifth nerve. A graphic reconstruction of this nerve showing its contribution to both limbs is given in Fig. 9. In all of the other cases sectioned, the fifth nerve followed its typical course to the normal intact limb.

The distance between the fully differentiated transplant and the normal limb varied much more in this series than in the dorso-dorsal grafts. This is shown in Table II, *B*, in which it is seen that the final position of the grafted limb varied from two to four segments caudal to the normal. In spite of the fact that in four of the six cases the grafted limb was less than four segments caudal to the normal, only one received contribution from the fifth nerve (case 28).

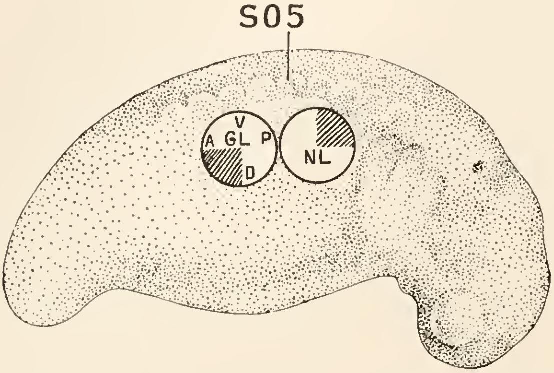


FIG. 4. Drawing of *Amblystoma* embryo showing supernumerary limb rudiment (*GL*) grafted (with inverted orientation) so as to center ventral to the seventh somite. The original antero-dorsal quadrant (shaded quadrant), which contains the bulk of the limb-forming cells, is brought into a postero-ventral position. *SO*5, fifth somite; *NL*, normal limb rudiment. × 12.

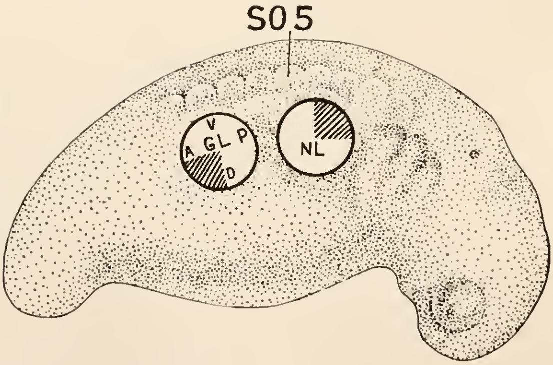
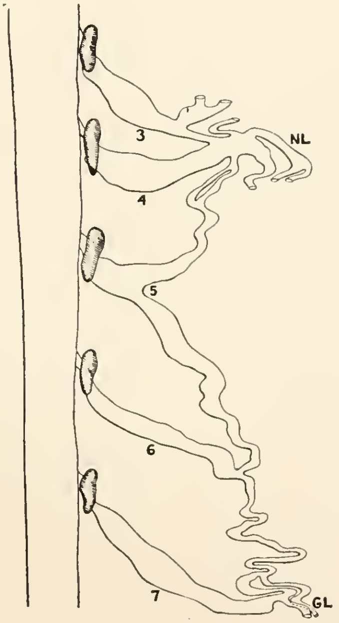


FIG. 5. Drawing of *Amblystoma* embryo showing supernumerary limb rudiment (*GL*) grafted (with inverted orientation) so as to center ventral to the eighth somite. *SO*5, fifth somite; *NL*, normal limb rudiment. × 12.

Although we did not study the nerve supply to any of the heterotopic limbs of the dorso-dorsal series which lacked coördinated function, we feel safe in assuming that such limbs were innervated entirely from the postbrachial region of the cord. In all previous experiments it has been found that whenever grafted limbs exhibit movements which are not coördinated with those of the normal intact limbs, they lack connection with the brachial reflex mechanism (Detwiler, 1920, 1925; Detwiler and Carpenter, 1929).

Discussion

The results of the experiments cited in this paper differ from those described in former communications (Detwiler, 1920, 1925) in showing that, when a supernumerary anterior limb bud is grafted just caudal to the normal intact limb rudiment, the most caudal of the normal brachial nerves (fifth) may (in some cases) be taken over by the graft rather than by the normal (Table II). This has occurred almost exclusively in limbs with dorso-dorsal orientation rather than in those in which the



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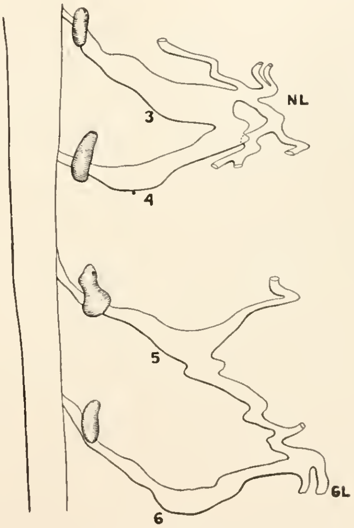

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FIG. 6. Graphic reconstruction of the segmental nerve supply to the normal limb (*NL*) and the grafted limb (*GL*) in case *HS*4–7. A supernumerary anterior limb rudiment was grafted (with dorso-dorsal orientation) so as to center ventral to the eighth somite (Fig. 3). × 20.

FIG. 7. Graphic reconstruction of the segmental nerve supply to the normal limb (*NL*) and the grafted limb (*GL*) in case *HS*4–49. A supernumerary limb was grafted (with dorso-dorsal orientation) so as to center ventral to the seventh somite (Fig. 2). × 20.

limb was inverted. In order to account for this difference, we have tentatively advanced the hypothesis that a stronger attractive influence upon the fifth (brachial) nerve is exerted by the graft when normally oriented, since, under these conditions, the bulk of the limb-forming cells in the grafted disc is closer to the growing fifth nerve than when the disc is inverted (Figs. 2 and 3; *cf.* Figs. 4 and 5).

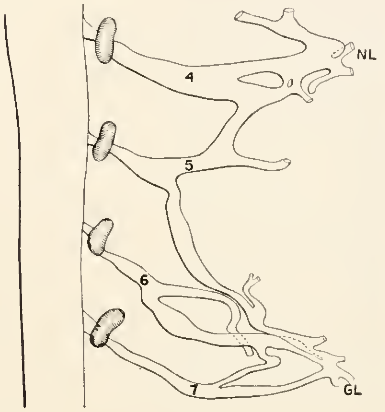
In the normal limb disc which extends from the anterior border of the third somite to the caudal border of the fifth, the cells may be said to be concentrated chiefly in the antero-dorsal quadrant (Fig. 2), even though the postero-dorsal quadrant has been shown by Swett (1923) to form a definite part of the limb. The antero-ventral and the postero-ventral quadrants contribute only very slightly to the limb (Swett, *op. cit.*).

When an additional rudiment is grafted just caudal to the normal so that it centers ventral to the seventh somite, the antero-dorsal quadrant of the graft is about as close to the outgrowing fifth nerve as is this quadrant in the normal appendage (Fig. 2). Consequently, as far as the distance factor is concerned, it seems not unreasonable to assume that the attraction of the graft on the fifth nerve is approximately as great as that exerted by the normal since, in the normal rudiment, the main mass of the limb-forming cells are concentrated beneath the third and the anterior half of the fourth somite.

When the graft is implanted so as to center ventral to the eighth somite there is a space between the two discs approximately the width of one somite (Fig. 3) and the antero-dorsal quadrant of the graft is further removed from the outgrowing fifth nerve than the corresponding quadrant of the intact limb. In spite of this, we found that in 7 cases out of 48 the fifth nerve grew caudally to the grafted limb. It must be remembered, however, that in the early growth of the embryo. the myotomes elongate in a caudo-lateral direction, which tends to mechanically influence the nerves to pursue a similar course, especially in their proximal portions. It seems not unreasonable to assume, therefore, that the fifth nerve, in its caudal growth to the graft, is aided initially by the characteristic caudal elongation of the myotomes. Only a very short growth in this direction would bring the growing nerve under the attractive influence exerted by the antero-dorsal quadrant of the graft. This is then apparently sufficient to insure its further growth to the grafted limb.

That the attraction of the normal limb rudiment, however, is typically greater is evident by the fact that in the majority of the cases, the fifth nerve supplies the orthotopic rather than the heterotopic appendage.

When the graft is inverted, its original antero-dorsal quadrant is brought into a ventro-caudal position and the distance between the concentrated mass of limb-forming cells and the outgrowing fifth nerve is greatly increased as compared with the distance in the dorso-dorsal orientations (*cf.* Figs. 4 and 5 with 2 and 3). It has been noted also from Table I that in the dorso-ventral series only one case was obtained in which the graft developed coördinated activities and in this case it was supplied by the fifth nerve (case 28, Table II). In many cases in this series, the graft was closer to the normal limb than in the dorso-dorsal series, yet no connection was made with the fifth nerve. It appears, therefore, that the failure of this nerve to supply the graft in the dorso-ventral combinations is associated with a more remotely located center of attraction as compared with the dorso-dorsal grafts.



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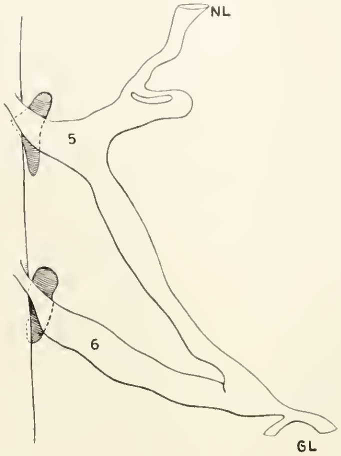

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Fig. 8. Graphic reconstruction showing segmental nerve supply to the normal limb (*NL*) and the grafted limb (*GL*) in case *HS*4–50. Conditions same as described for Fig. 6. × 20.

Fig. 9. Graphic reconstruction showing segmental nerve contribution to the grafted limb (*GL*) in case *HS*4–28. A supernumerary limb was grafted (with inverted orientation) so as to center ventral to the seventh somite (Fig. 4). × 20.

Although the interpretation which we have pictured is largely hypothetical for the present results, we do have considerable evidence from former experiments (Detwiler, 1920, 1922, 1925) to indicate that the limb rudiment does attract spinal nerves, and it seems not unreasonable to suppose that when the grafted limb is inverted, as in the present experiments, the distance between the outgrowing fifth nerve and the bulk of the limb-forming cells is so greatly increased, that this nerve falls entirely under the influence of the more closely situated attraction center represented by the normal limb.

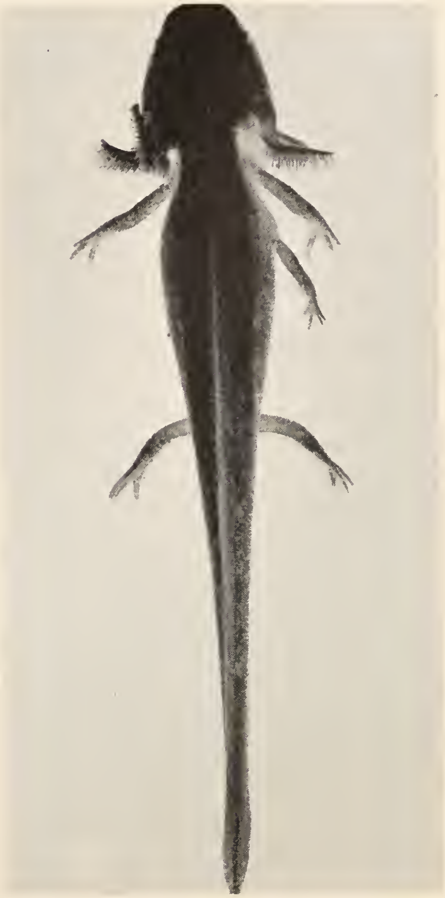

FIG. 10. Photograph of *Amblystoma* larva *HS*4–37 showing supernumerary limb grafted four segments caudal to the intact normal limb. × 2.7.

SUMMARY

1. When a supernumerary anterior limb disc is grafted (with normal orientation) caudal to the intact rudiment, so as to center ventral to the seventh or eighth somites (Figs. 2, 3), the fifth nerve, which normally supplies the orthotopic limb, may grow caudally to the graft. This has been found to occur in seven out of forty-eight cases. The entire nerve may be taken over by the graft (Fig. 7) or it may bifurcate so as to send one branch to the normal and the other to the supernumerary limb (Figs. 8, 9).

When the graft is supplied by the fifth nerve its activities are coördinated with those of the normal limbs. The movements of the muscles in the grafted appendage are synchronous with those in the homologous muscles of the normal limb of the same side.

2. When the supernumerary limb disc is grafted to the same position, but with inverted orientation (dorso-ventral, Figs. 4, 5), there is a greatly reduced number of cases in which the fifth (brachial) nerve supplies the graft. In fact in only one case in fifty-eight was this condition obtained.

3. The greater number of cases with brachial nerve contribution (fifth nerve) to the supernumerary limb in the experiments with normally oriented (dorso-dorsal) grafts, as compared with those which have been inverted (dorso-ventral), is interpreted as due to the fact that a stronger attraction is exerted upon the fifth nerve. The bulk of the cells in the limb disc is concentrated in the antero-dorsal quadrant (Fig. 2). When the graft is inverted, the antero-dorsal quadrant occupies a caudo-ventral position and the distance between the outgrowing fifth nerve and the concentrated mass of limb cells is thus considerably increased (*cf.* Figs. 4, 5 with 2 and 3). Any attractive influence of the graft upon the fifth nerve, therefore, cannot be realized by reason of the distance factor.

4. More evidence has accumulated in the present experiments to bear out former conclusions (Detwiler, 1925, Detwiler and Carpenter, 1929) that when grafted anterior limbs exhibit movements which are coördinated with those of normal intact anterior limbs, they have some connection with the central brachial reflex correlation mechanism.

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